STUDIES ON THE URINARY C_{21} ADRENOCORTICAL STEROIDS.

by

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Contents

3, 4. Optimum /

SECTION 1.

GENERAL INTRODUCTION.

GENERAL INTRODUCTION.

Interest in the therapeutic value of cortisone and other adrenocortical steroids has stimulated research into the biochemistry of these compounds in recent years. Such studies depend to a large extent on effective methods for the estimation of microgram amounts of adrenocortical steroids in biological materials; clinically, the estimation of these steroids and their metabolites in urine is of particular importance.

1,1. CHEMICAL NATURE OF C_{21} ADRENOCORTICAL STEROIDS.

Prior to the isolation of C_{21} adrenocortical steroids from urine their presence in urine was inferred since extracts could be prepared having the characteristic biological activity of such steroids. Also, methods of chemical estimation were developed for C_{21} adrenocortical steroids and applied to the investigation and estimation of such compounds and their metabolites in urine. The assumption that there were adrenocortical steroids in urine has been confirmed by the isolation of such compounds in recent years (Appendix I).

The C_{21} steroids isolated from the adrenal gland contain 3 to 5 oxygen atoms [x] present as hydroxyl- or carbonyl-groups. /

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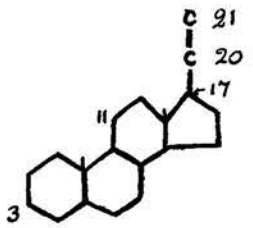
[x] except progesterone, $C_{21}O_2$; this steroid will not be considered as a typical adrenocortical steroid for the purposes of work described in this Thesis.

groups. The positions of substitution of these oxygen atoms are C3, C11, C17, C20 and C21 in the steroid nucleus:

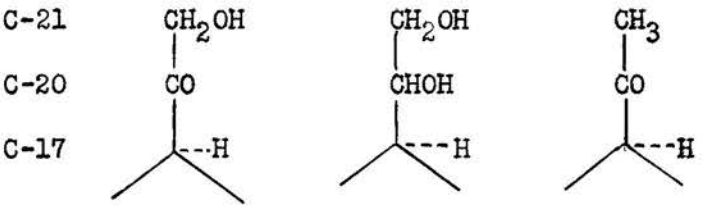


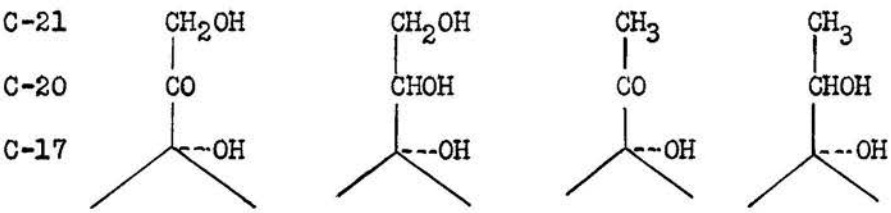
C-3 and C-11 substituents:

1. αβ unsaturated ketone at C-3;
2. secondary alcohol at C-3;
3. carbonyl group at C-11;
4. secondary alcohol at C-11.

C-17, C-20 and C-21 substituents.

These can be illustrated diagrammatically as follows:





Stereochemical configurations. In the naturally occurring adrenocortical steroids a hydroxyl group at C-11 is invariably β-orientated; at C-17 a hydroxyl group or a hydrogen atom has an α- and the side chain a β-configuration. Both 3α- and 3β-hydroxy compounds, and pregnane and allopregnane derivatives, occur.

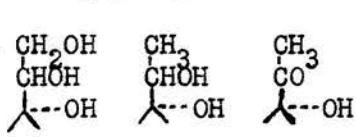
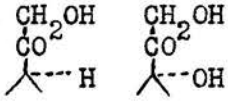
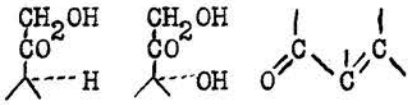
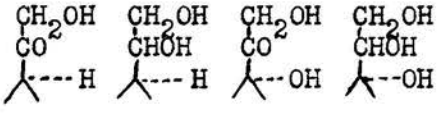
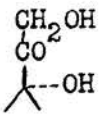
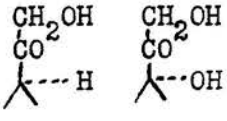
Amongst/

Amongst and in addition to the expected characteristic chemical properties of these functional groups are some of special interest to the detection and estimation of the C_{21} adrenocortical steroids. Due to steric hindrance, a carbonyl group at C-11 (in contrast to one at C-3 or C-20) is inert to reagents such as hydroxylamine, phenylhydrazine, and Girard's reagents (Girard and Sandulesco, 1936). For the same reason, an 11β- or 17α-hydroxyl group can not be acetylated; the 11α- or 17β-configurations which are easily acetlyated have not been found in any of the naturally-occurring C_{21} adrenocortical steroids. Steroids having C-11 and C-17 hydroxyl groups are readily dehydrated by acid, the former giving compounds unsaturated at C-9:11. A C-17 side chain is easily removed by chromic acid if a C-17 hydroxyl group is present. An α-ketol group in the side chain is alkali sensitive. This lability of compounds with these groupings is a major obstacle to their quantitative estimation in, and isolation from, urine.

The αβ unsaturated 3-ketone group absorbs strongly light of wavelength about 245mμ; it reduces phosphomolybdic and arsenomolybdic acids (Heard & Sobel, 1946). The double bond may be hydroxylated with osmium tetroxide (Manaro, ~~Morato~~ & Zygmuntowicz, 1951), or with potassium permanganate, and may form addition compounds with halogens.

Alkaline silver nitrate, cupric ions and phosphomolybdic acid are readily reduced by a primary α-ketol side chain which/

TABLE 1. Chemical methods for the estimation of C_{21}-adrenocortical steroids.

Reacting groups	Principle of method	Authors
1. 	estimation of 17-ketosteroids formed on oxidation with periodic acid	Talbot & Eitingon (1944)
2. 	reduction of cupric ions in alkaline solution	Talbot, Saltzman, Wixom & Wolfe (1945)
3. 	reduction of phosphomolybdic acid	Heard & Sobel (1946)
4. 	estimation of formaldehyde formed on oxidation with periodic acid	A. Lowenstein, Corcoran & Page (1946) B. Daughaday, Jaffe & Williams (1947)
5. 	reaction with phenylhydrazine in concentrated sulphuric acid	Porter & Silber (1950)
6. 	reduction of alkaline ferricyanide	Gordon & Pelly (1951)

Notes: a. In methods 4A and 4B, formaldehyde is formed on periodate oxidation of the steroids; these methods thus measure 'formaldehydogenic' steroids. Similarly, methods 2, 3, and 6 measure 'reducing' steroids.

b. The action of periodic acid on steroids with all the C-17 side chains listed in methods 1 and 4 was not investigated by the original authors but may be deduced from the known behaviour of such compounds in preparative work (review: Fieser & Fieser, 1949; see also Fig. 1).

c. Fieser, Fields & Lieberman (1944) originally suggested the use of periodic acid in the estimation of adrenocortical steroids.

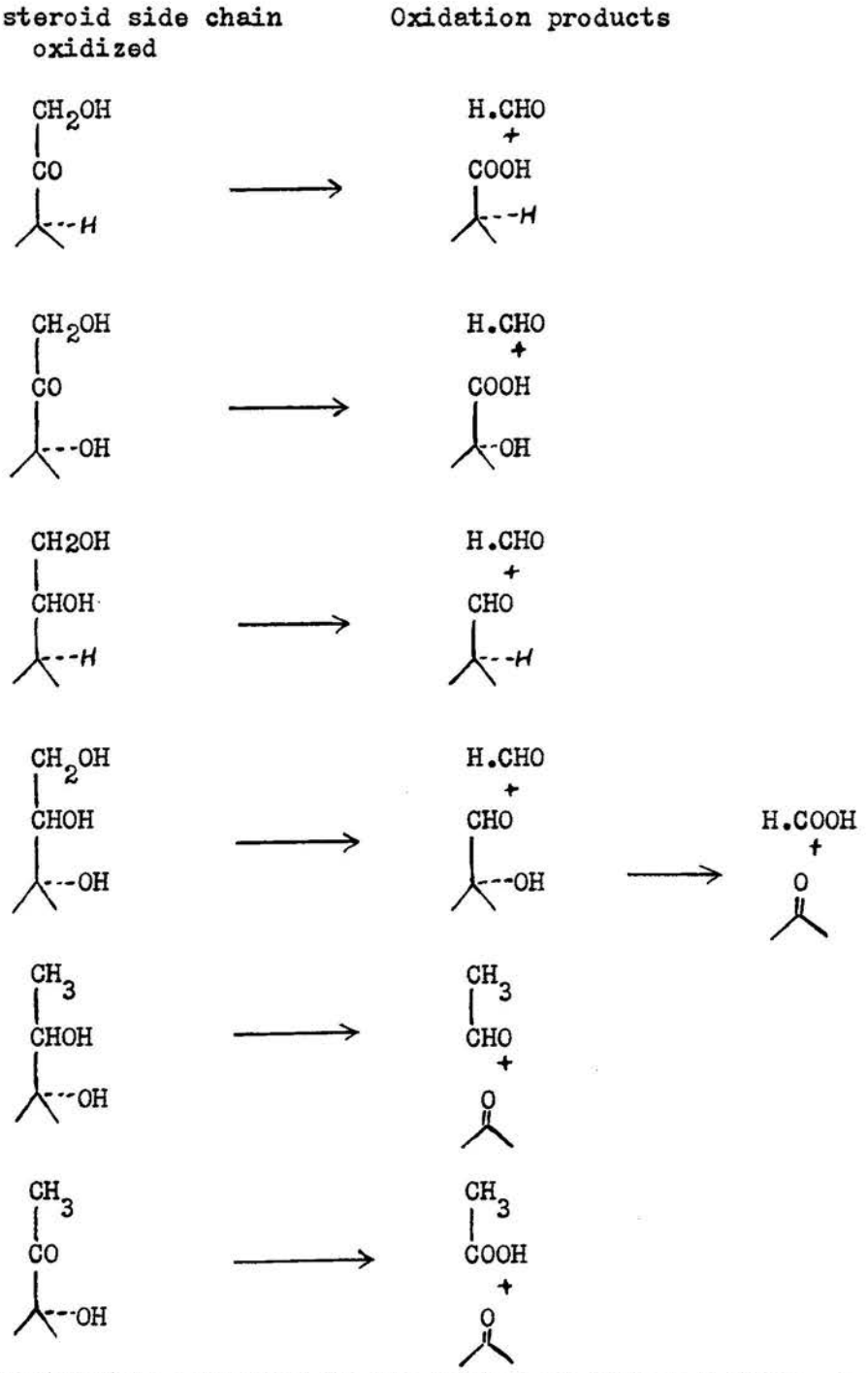
which also reacts with periodic acid resulting in fission of the C-20 - C-21 bond. Reaction with periodic acid and lead tetra-acetate leads to fission of C-C bonds in 17:20- and 20:21-glycols. Fieser & Fieser, (1949) give the original references to such properties and reactions of C_{21} adrenocortical steroids.

1,2. CHEMICAL ESTIMATION OF C_{21} ADRENOCORTICAL STEROIDS AND OF URINARY CORTICOIDS.

It is generally believed that adrenocortical steroids having "cortin-like" activity contain (a) an $\alpha\beta$ unsaturated 3-ketone group (see, however, Tait, Simpson & Grundy, 1952), (b) a primary α-ketol group in the C-17 side chain and also may have (c) a tertiary C-17 hydroxyl and (d) a C-11 oxygen function. The chemical methods which have been developed were designed primarily to estimate these physiologically active steroids; most chemical methods, however, may also estimate physiologically inactive C_{21} steroids. The chemical methods which have been developed and applied to the estimation of urinary C_{21} adreno-cortical steroids or their C_{21} metabolites (corticoids) are summarized in Table 1.

All of the methods are dependent on colorimetric reactions. Of general application to (steroid) secondary alcohols is the colorimetric estimation of hemidinitrophthalates in alkaline solution (Engel, Patterson, Wilson & Schinkel, 1950). The polarographic technique of Wolfe, Hershberg & Fieser (1940) for ketosteroids does not appear to have been used extensively (c.f. Butt, Morris, Morris & Williams, 1951).

FIG. 1. Oxidation of C-17 steroid side chains with periodic acid (Fieser & Fieser, 1949, for original references).



17-ketosteroids estimated in the method of Talbot & Eitingon (1944). Formaldehyde estimated in the methods of Lowenstein et al.(1946), Corcoran & Page (1948), and Daughaday et al. (1947, 1948).

The method selected for work reported in this Thesis was based on that described by Daughaday et al.(1947, 1948). Later, after the development of a method for estimating steroids with a $CH_3.CHOH.C<$---OH C-17 side chain, (Section 3) a combined method for estimating 'formaldehydogenic' and 'acetaldehydogenic' steroids was used. Although the formaldehydogenic steroid method may estimate four types of C-17 side chain, including those which may be associated with biologically inactive compounds (having a 20-hydroxyl group), the method would seem to be more sensitive, reproducible, and less liable to interference from non-steroidal material than the 'reducing steroid' methods. A further advantage of the formaldehydogenic method is that a strict stoichimetric relationship exists, between steroid oxidised and formaldehyde evolved (c f. Mason, 1951). The action of periodic acid on the various C-17 steroid side chains is illustrated in Fig. 1.

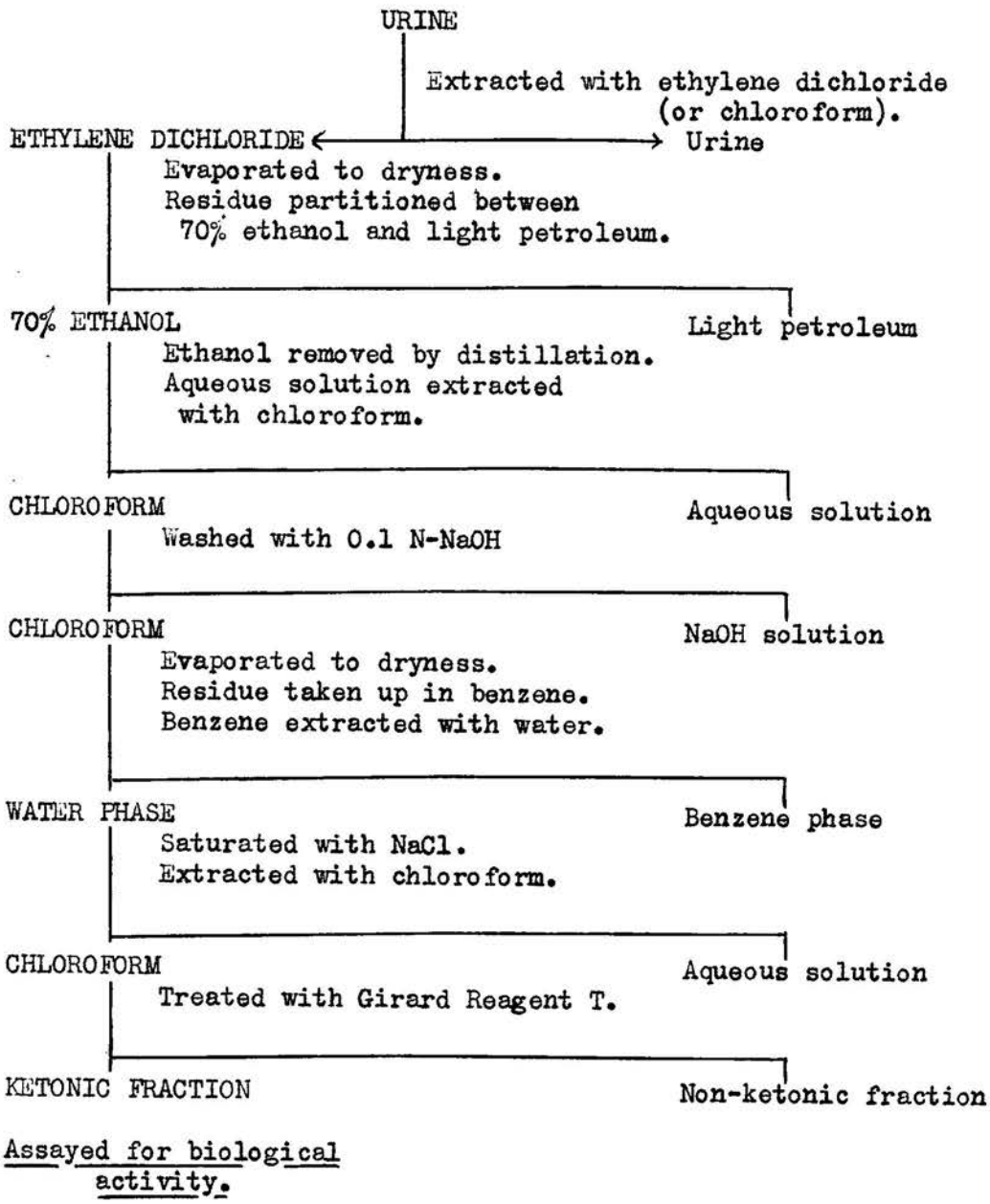
These methods of chemical estimation are quantitative for pure steroids. Their accuracy when applied to urine extracts is controlled by two factors:

(a) The efficiency of the hydrolytic procedure used to liberate the free steroids from their water-soluble conjugates in urine.

(b) The efficiency of the extraction and purification of the free steroids subsequent to the preliminary hydrolysis.

It may be noted that little has been done on the quantitative determination in urine of the conjugates themselves. 1,3./

FIG. 2. Extraction of 'cortin' from urine (Venning et al.,1944).



1,3. EXTRACTION OF CORTICOIDS FROM URINE AND PURIFICATION OF EXTRACTS.

Perla & Marmorsten-Gottesman (1931) showed that a benzene extract of normal human adult's urine (not subjected to a preliminary hydrolysis) raised the resistance of adrenalectomised rats to histamine poisoning. Subsequent workers confirmed the 'cortin-like' activity of urine extracts (Grollman & Firor, 1932-33; Weil & Browne, 1939; Dorfman, Horwitt & Fish, 1942; Venning, Hoffman & Browne, 1944). After it had been shown that the hormones of the adrenal cortex were steroids (review: Reichstein & Shoppee, 1943) a more rational approach to the extraction and purification of possible urinary steroids of adrenal origin could be undertaken. Venning et al. (1944) outlined a method for purifying and concentrating biologically active 'cortin' from urine of human subjects. The bioassays employed were (a) the protection of adrenalectomised rats against the lethal action of low temperatures (b) the sustaining of life and growth in adrenalectomised rats and (c) the deposition of glycogen in the liver of fasted adrenalectomised rats. An outline of their purification is given in Fig. 2. Relatively little biological activity was lost in each stage of the purification.

Subsequent workers employing biological- or chemical-assay methods have used extraction and purification techniques based on the same principles; more recently adsorption and partition chromatography have been applied as a final purification stage

and/

TABLE 2. Methods used for the hydrolysis of urinary corticoid conjugates and the extraction and purification of urine extracts; corresponding recoveries of steroids added to urine

Authors	Methods used for the hydrolysis of urinary conjugates	Solvent used for extraction of free steroids from urine
Talbot & Eitingon (1944)	1. Heat at pH 5.8-6.2 [x] 2. Incubation with crude enzyme preparations [x] 3. Hot 15% HCl [x]	Ethyl acetate
Talbot, Saltzman, Wixom & Wolfe (1945)	None	Chloroform
Lowenstein, Corcoran & Page (1946) Corcoran & Page (1948)	pH 1, unspecified time	Chloroform
Heard, Sobel & Venning (1946)	pH 1, 0 hr.	Ether-chloroform (4:1)
Daughaday, Jaffe & Williams (1947, 1948)	pH 1.7, unspecified time	Chloroform
Tompsett & Oastler (1947)	None	Chloroform
Lloyd & Lobotsky (1950)	pH 1, unspecified time	Chloroform
Sprechler (1950)	pH 1, 0 hr.	Chloroform
Staudinger & Schmeisser (1950)	acidified with acetic acid	Chloroform
Porter & Silber (1950)	None	Chloroform
Hollander, DiMauro & Pearson (1951)	pH 1, 24 hr., room temperature	Chloroform
Hioco & Samter	pH 1, 0 hr.	Chloroform

[x] Hydrolysis of substances in NaOH-washed n-butanol extracts of urine.

Stages employed in purification of extracts of urine	Steroids added to urine: Compound	Recovery (%)
NaOH wash; Girard separation (ketonic fraction taken)		
NaOH wash; benzene-water partition; Girard separation (ketonic fraction taken)	17-hydroxycorticosterone 17-hydroxy-11-dehydrocorticosterone 11-dehydrocorticosterone Corticosterone	113, 114 71, 129 52, 51 50 - 59
NaOH wash	Adrenocortical extract	90-104
NaOH wash	11-deoxycorticosterone	90-104
Benzene-water partition		
NaOH wash; Girard separation (ketonic fraction taken)		
NaOH wash; benzene-water partition		
NaOH wash; Girard separation (ketonic fraction taken)	11-deoxycorticosterone 17-hydroxy-11-dehydrocorticosterone 17-hydroxy-11-dehydrocorticosterone acetate	102-108 97, 101 94, 100
NaOH wash; 70% ethanol-light petroleum partition	11-deoxycorticosterone acetate and glucoside	100 ± 5
None	17-hydroxy-11-dehydrocorticosterone	85-91
NaOH wash	xx	
NaOH wash; benzene-water partition		

[xx] Recoveries of 11-deoxycorticosterone added to urine extracts reported as varying according to the purification used in preparing the extract (cf. Sections 3, 1 and 3, 7).

and for the separation of individual compounds (Section 5). At present quantitative data on the efficiency of extraction and purification techniques are available for only a few C_{21} adrenocortical steroids, and this problem is in urgent need of investigation, particularly in the case of the more highly oxygenated or polar [x] compounds. It may not be practical to develop a single purification procedure for all the urinary corticoids since the range of their polarities is wide. In Table 2 methods used by various workers in the estimation of corticoids are summarized; the reported recoveries of steroids added to urine are noted.

Extraction of free corticoids from urine: chloroform, ethylene dichloride, ether and benzene (in decreasing order of efficiency for 11-deoxycorticosterone - Heard, Sobel & Venning, 1946 - or for 17-hydroxycorticosterone and 17-hydroxy-11-dehydrocorticosterone - Talbot et al. 1945) have been used. Extractions may be carried out in continuous extractors, or by multiple contact extraction in separating funnels. The more polar, highly oxygenated corticoids may not be extracted efficiently, especially in the latter type of technique. It is notable that a large proportion of urinary corticoid material seems to be the polar pregnane-3α:17α:21-triol-11:20-dione (Baggett, Glick & Kinsella, 1952). Romanoff, Wolf & Pincus (1952) identified/

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[x] The use of the term 'polar' is discussed in Appendix II.

identified allopregnane-3β:11β:17α:21-tetrol-20-one in urine of normal subjects. Thus, even if multiple contact extraction is efficient for $C_{21}O_3$, $C_{21}O_4$ and some $C_{21}O_5$ steroids it should be borne in mind that few data are available to indicate that it is efficient for the extraction of more polar compounds.

Purification of chloroform extracts of urine by washing with NaOH solutions: the use of an alkali wash has the disadvantages that polar corticoids may be lost and (possibly) sensitive α-ketol groups destroyed. Back-washing the NaOH and water washes with fresh solvent may overcome the former, and chilling the solutions may minimise the latter.

Benzene - water partition: this technique is based on the observations of Pfiffner & Vars (1934), and Mason, Myers & Kendall (1936) in their work on the isolation of steroids from adrenal glands. Talbot et al. (1945) used a benzene - water partition because "only corticosteroids with an oxygen on the 11th carbon atom tend to pass readily from benzene to water under the conditions used". Thus Talbot et al. found that when benzene solutions of steroids were extracted with water (10 x 1 vol.) the percentages recovered from the aqueous extract were:

	Steroid in aqueous phase (%)
17-hydroxycorticosterone	100
17-hydroxy-11-dehydrocorticosterone	100
11-dehydrocorticosterone	24
corticosterone	38
11-deoxycorticosterone	2
pregn-4-en-3-one-17:20:21-triol	(almost) 100

From/

From these results it appears that no sharp separation is obtained in such a multiple contact extraction and it is of doubtful value to use the system. Some workers however have used the technique (Table 2). A true counter-current distribution technique such as that used by Craig (1944) might give sharp separations between individual steroids or groups of steroids, but probably would be too cumbersome for quantitative studies. Simpler techniques are offered by partition chromatography (Section 5).

Partition between aqueous ethanol and light petroleum or hexane: such partitions, removing relatively non-polar substances from extracts containing adrenocortical steroids have not been widely used for urinary studies (Venning et al., 1944); they have been more widely employed in the estimation of such steroids in blood (Corcoran & Page, 1948; Nelson & Samuels, 1952; Bush, 1952) where lipoid impurities are relatively large. These workers obtained no indication of appreciable losses of adrenocortical steroids in such partitions, and as shown in Section 7 considerable amounts of contaminants may be removed thus from urinary corticoid concentrates.

Separation of ketonic and non-ketonic substances (Girard & Sandulesco, 1936): this separation may be of value where estimation of the biologically active adrenocortical steroids is desired. However many urinary corticoids appear to be non-ketonic (Appendix I) and estimation of the ketonic fraction only of urine/

of urine extracts may bear no relation to the total corticoid level. In all the methods noted in Table 2 where a Girard separation is carried out, only compounds in the ketonic fractions are assayed. Some of the corticoids in crude urine extracts may not be stable to the conditions necessary for the formation and acid hydrolysis of Girard complexes (c f. Section 4,5 and 4,10), although Talbot et al. (1945) obtained 81 - 94% recovery of 17-hydroxycorticosterone, 17-hydroxy-11-dehydrocorticosterone, 11-dehydrocorticosterone and corticosterone added to urine extracts before Girard separation.

Chromatographic separation and purification: partition chromatography offers great advantages as a method of purification, especially for quantitative work; the first successful techniques for the adrenocortical steroids were developed by Zaffaroni, Burton & Keutman (1949, 1950). Even with these techniques it is not possible to apply a single partition system to the whole range of corticoids from the least to the most polar. Chromatography will be considered in detail in Section 5.

1,4. HYDROLYSIS OF URINARY CORTICOID CONJUGATES.

Some urinary steroids are known to be excreted as water-soluble conjugates. Thus, from the urine of human subjects have been isolated glucuronides of oestriol (Cohen & Marrian, 1936), pregnane-3α:20α-diol (Venning & Browne, 1936; Venning, Henry & Browne, 1937; Sutherland & Marrian, 1947), pregnan-3α-ol-20-one/

pregnan-3α-ol-20-one (Marrian & Gough, 1946), and an apparent mixture of the glucuronides of pregnane-3α:20α-diol and pregnane-3α:17α:20α-triol (Mason & Kepler, 1945). Sulphates of androsterone (Venning, Hoffman & Browne, 1942) and dehydroepiandrosterone [x] (Munson, Gallagher & Koch, 1944) have been obtained. Methods for the quantitative estimation of oestrogens and androgens were delayed because the importance of such conjugates was not realised at first; it now appears that these groups of steroids are excreted mainly in the form of water-soluble conjugates which may be hydrolyzed to yield the free steroids by heating in acid solution, or by incubation with enzyme preparations (review: Marrian & Bauld, 1951).

Hot acid hydrolysis may be destructive of urinary corticoids (Heard, Sobel & Venning, 1946) but acid treatment at room temperature, and more recently, incubation with enzyme preparations have been used for the hydrolysis of possible conjugated corticoids in urine.

Acid hydrolysis of corticoid conjugates: Venning, Kazmin & Bell (1946) using a biological assay for material promoting glycogen deposition in the livers of fasting animals observed that an increased amount of active material could be extracted from urine after acidification of the urine to pH 1; further small amounts of active substances were extractable after allowing the acidified urine to stand for 24 hr. at room temperature.

These/

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[x] isolated as the semicarbazone.

These workers suggested that at least part of the active 'cortin' was present in a conjugated form. Heard et al. (1946) obtained similar effects when estimating the reducing steroids in urine. In addition they found that neutralization of the urine after acidification decreased the amount of reducing steroid extractable to about the level obtained for unacidified urine. Heard et al. considered that the effects were due either to hydrolysis of water-soluble conjugates, or to more complete extraction from acidified urine, concluding that the latter was true of a larger part of the reducing substances. They also noted that after 24 hr. at pH 1, room temperature, neutralization of the urine did not affect the amount of reducing steroid extractable (Venning, private communication to Professor Marrian). Unfortunately, this observation was not published, for, taken in conjunction with the other reported behaviour of the reducing steroid fraction it was the first definite evidence for the presence of corticoid conjugates in urine.

Without thorough investigation of the optimum conditions many workers adopted empirical procedures for acid treatment of urine prior to extraction of corticoids. Thus, urine samples were acidified to various pH values between pH1 and pH 2 ; often the time between acidification and extraction was not specified - the importance of this factor did not seem to be appreciated. Other workers extracted urine samples immediately after acidification or after a definite time interval, usually/

usually 24 hr. (Table 2). A more systematic study of acid hydrolysis has been carried out in this laboratory (Paterson, 1950; Paterson, Cox & Marrian, 1950; Paterson & Marrian, 1951) and part of this work, carried out by the author, is detailed in Section 4.

Hydrolysis with enzyme preparations rich in β-glucuronidase.

The advantages of other methods, less destructive than acid treatment for the hydrolysis of urinary conjugated corticoids have been realised recently. The hydrolysis of urinary steroid glucuronides by crude β-glucuronidase preparations has been known for some time, although the difficulties inherent in the use of enzymic hydrolysis for quantitative work had confined its application almost entirely to preparative work until 1949.

In 1933, Marrian observed that bacterial action in pregnancy urine hydrolyzed the conjugated oestrogens (mainly oestriol glucuronide). For the isolation of C_{19} and C_{21} steroids from urine, rat liver preparations containing β-glucuronidase have been used (Talbot, Ryan & Wolfe, 1943; Mason & Kepler, 1945; Mason & Strickler, 1947; Mason, 1948; Mason & Schneider, 1950). β-Glucuronidase concentrates from calf- or ox-spleen have been used in the preparation from urine of oestriol (Grant & Marrian, 1950), and oestrone (Oneson & Cohen, 1951). Preparations of bacterial origin and preparations from ox-spleen have been used in the isolation of androst-16-en-3α-ol from urine (Brooksbank & Haslewood, 1950, 1952).

Enzymic/

Enzymic hydrolysis was applied to the estimation of oestrogens in pregnancy urines by Patterson (1937) using as a source of the enzyme Escherichia coli cultures. Odell, Skill & Marrian (1937) demonstrated the hydrolysis of pure sodium oestriol glucuronidate by a mouse intestinal extract. Preparations of ox-spleen β-glucuronidase were shown by Fishman (1939) to hydrolyze pure sodium oestriol glucuronidate. Buehler, Katzman, Doisy & Doisy (1949) used β-glucuronidase preparations from Escherichia coli cultures in developing techniques aimed at the quantitative hydrolysis of the urinary glucuronides of oestrogens, pregnane-3α:20α-diol and ketosteroids. Bitman & Cohen (1950) have also studied the hydrolysis of ketosteroids with ox-spleen enzyme.

By analogy with other steroids it was conceivable that corticoids would be excreted in part as glucuronides, and recently workers have investigated the effect of β-glucuronidase on the amount of corticoids extractable from urine. With the oestrogens, ketosteroids and pregnane-3α:20α-diol, enzymic hydrolysis yields amounts of free steroid of the same order as that obtained by hot acid hydrolysis. However, enzymic hydrolysis appears to liberate amounts of free corticoid ('reducing' or 'formaldehydogenic') many times that obtainable by acid hydrolysis under optimum conditions. Such results were obtained independently by several workers including the author, so consideration of enzymic hydrolysis of urinary corticoid conjugates is given in Section 4.

1,5./

1,5. Purpose of the Investigation

The objective was the quantitative estimation of C_{21} steroids of adrenocortical origin in urine. A new method for the estimation of a group of C_{21} adrenocortical steroids was developed and applied to urinary studies. As is indicated in this Introduction, the major obstacles to quantitative estimations of urinary corticoids are the hydrolysis of the steroid conjugates and the efficient purification of urine extracts containing the free steroids. Both of these problems have been investigated. Proof that compounds released after various hydrolytic treatments are indeed C_{21} steroids of adrenocortical origin depends on isolation studies. Some experiments have been conducted with this objective; pregnane-3α:17α:20α-triol has been isolated from the urine of normal subjects for the first time.

SECTION 2.

METHODS GENERALLY USED.

METHODS GENERALLY USED.

The methods described here are those which have been used generally in the work reported in this Thesis. Any variations in procedure are detailed under the particular experiments concerned.

2,1. Apparatus. All glass apparatus was used, with standard ground glass Quickfit Quartz joints. No grease was used on taps or joints; where necessary these were lubricated with a small amount of the liquid being used.

2,2. Collection of Urine. Urine specimens were collected over 24 hr. with 1-2 ml. chloroform as preservative. The subjects were normal men.

2,3. Measurement of pH of solutions. A Cambridge Instrument Co. pH meter with glass electrode was used for all pH measurements.

2,4. Hydrolysis of urinary steroid conjugates; acid hydrolysis. Measured portions (50-100 ml.) of a 24 hr. specimen were acidified to pH 0.95-1.1 with 10 N-H_2SO_4. After 24 hr. at 25° the acidified urine was extracted. For a 'hydrolysis curve' (see Section 4) a larger portion of a 24 hr. specimen was acidified to allow a number of samples to be withdrawn for analysis at various time intervals.

2,5./

2,5. Hydrolysis of urinary steroid conjugates; enzymic hydrolysis.

(a) Preparation of β-glucuronidase concentrates.

Based on the observations of Graham (1946), Mills (1948) and Kerr & Levvy [x] (1951) the following method was used. Finely minced fresh ox-spleen was suspended in 0.1 N-acetate buffer pH 5.2 and allowed to stand at 37° for 4 hr. After centrifuging off tissue debris, the supernatant was made 60% saturated with ammonium sulphate and the mixture left at 0° overnight. The precipitate, stirred up in the minimum volume of water was dialysed almost completely free of ammonium sulphate against running tap water in 4 hr. The solution was then made 25% saturated with ammonium sulphate and kept a few hours at 0°. After centrifuging, the precipitate was rejected and the supernatant made up to 50% saturated with ammonium sulphate. The precipitate which formed on standing overnight at 0° was centrifuged down, dialysed free of ammonium sulphate and stored in the refrigerator. These crude enzyme preparations generally retained their activity for about a month, after which rapid deterioration was frequently noted.

(b) Estimation of activity of enzyme.

The method of Kerr, Graham & Levvy (1948) was used, except that acetate buffer replaced the citrate buffer. To two 10 ml. centrifuge tubes containing 0.2 ml. 0.1 M A.R. acetate buffer pH 5.2, were added 0.2 ml. 0.06 M-phenylglucuronide (at pH/

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[x] Also private communication to Dr. Sutherland prior to publication of results.

pH 5.2) and 0.4 ml. of the enzyme preparation (or an appropriate dilution of the preparation). Similar tubes were made up containing no substrate (enzyme blank), no enzyme (substrate blank) and no substrate or enzyme (reagent blank). Water was added to these blank tubes to make up the total volume to that of the other estimations (0.8 ml.). The tubes were incubated in a water bath at 37° for 1 hr. The reaction was stopped by the addition of Folin-Ciocalteu reagent (B.D.H. reagent diluted 1 in 5). The contents of the tubes were mixed, and after centrifugation 2 ml. aliquots of the supernatant solutions were pipetted into tubes containing 4 ml. of 10% Na_2CO_3 A.R. After mixing the tubes were kept at 37° for 20 mins. for the colour development. The solutions were read against the reagent blanks in the Spekker photoelectric absorptiometer using Ilford No. 602 filters. After correcting for the enzyme and substrate blanks, the amount of phenol liberated by the enzyme was obtained by reference to a phenol calibration curve constructed under the same experimental conditions. The activity of the enzyme was expressed in glucuronidase units (G.U.) per ml. where 1 G.U. is the amount of enzyme which will liberate 1 µg. of phenol in one hour under the conditions described.

A single ox-spleen processed as described above generally yielded a final solution containing a total of from 20,000 - 80,000 G.U.(average about 40,000 G.U.).

(c) Enzymic hydrolysis of urine or urine extracts.

Initially the hydrolyses were carried out at pH 5 by analogy/

analogy with the conditions required for pregnane-3α:20α-diol glucuronide (Sutherland, 1950), but after experiments to determine the pH optimum of the reaction with urinary substrates yielding acetaldehydogenic and formaldehydogenic substances (4,7) a pH of 4.5 was selected.

An arbitrary enzyme concentration of 10,000 G.U./l. urine was used. This concentration was found by Sutherland (1950) to give 78 - 96% hydrolysis of pregnane-3α:20α-diol glucuronide added to urine, in 3 hr. at 37° (hot acid hydrolysis taken as 100%). Procedure for carrying out enzymic hydrolysis. Urine or extracts of urine in aqueous solution were acidified to pH4.5 with glacial acetic acid A.R. The solution was made approximately 0.1 N in acetate buffer pH 4.5 by adding a concentrated buffer solution, and the calculated amount of enzyme solution mixed in (the activity of stored enzyme solutions was checked regularly, and usually just before use). The mixture was then incubated at 37°, for 24 hr. After incubation the solutions were cooled rapidly to room temperature by putting them in the refrigerator, and then extracted with chloroform in the usual way.

2,6. Extraction of urine. Urine samples were extracted three times with 2 volumes of chloroform. Using these proportions, emulsions were rarely obtained. The chloroform extracts were washed once with 0.1 vol. 0.1 N-NaOH and twice with 0.1 vol. water. The water-washings were back washed once with 2 vols. chloroform, this chloroform being added to the main chloroform extract. The chloroform extracts were dried with the minimum amount/

amount (about 2 g./100 ml.) of A.R. anhydrous sodium sulphate for 20 min. After filtering off the sodium sulphate, the chloroform extracts were evaporated to dryness under reduced pressure in a water bath at about 60°.

2,7. Estimation of adrenocortical steroids. The method detailed in Section 3 for estimating acetaldehydogenic and formaldehydogenic steroids was used except for some earlier experiments reported in Section 4A.

2,8. Preparation of standard solutions of pure steroids. All steroids were dried in vacuo over P_2O_5 before weighing. Solutions were prepared in redistilled ethanol.

SECTION 3.

A METHOD FOR THE ESTIMATION OF C_{21} 17:20-DIHYDROXY-20-METHYLSTEROIDS, AND ITS APPLICATION TO URINE EXTRACTS.

TABLE 3. 20-methylsteroids, probably of adrenal origin, isolated from human urine

Compound	Associated condition of subjects	References
Pregnane-3α:17α:20α-triol	Adrenal hyperplasia	Butler & Marrian (1937, 1938)
	Adrenal hyperplasia Adrenocortical tumour	Mason & Kepler (1945)
	Adrenal hyperplasia	Miller & Dorfman (1950)
Pregnane-3α:17α-diol-20-one	Adrenal hyperplasia and adrenocortical tumour	Lieberman & Dobriner (1945)
	Adrenal hyperplasia	Miller & Dorfman (1950)
Pregn-5-ene-3β:17α:20α-triol	Adrenocortical tumour	Hirschmann & Hirschmann (1950)
Pregn-5-ene-3β:17α-diol-20-one	Adrenocortical tumour	Hirschmann & Hirschmann (1947)
Pregnane-3α:20α-diol-11-one	Adrenal hyperplasia	Lieberman, Fukushima & Dobriner (1948a, 1950)
Pregnan-3α-ol-11:20-dione	Adrenal hyperplasia	Lieberman *et al.* (1950)
Pregnane-3α:17α-diol-11:20-dione	After administration of ACTH and cortisone to patients with neoplasia	Lieberman, Hariton, Stokem, Studer & Dobriner (1951)

3, 1. INTRODUCTION

Hyperactivity of the adrenal cortex in human subjects due to malignant neoplasm, hyperplasia or stimulation by adrenocorticotropic hormone (ACTH) has frequently been observed to be associated with the urinary excretion of considerable amounts of various C_{21} 20-methylsteroids (Table 3).

Such urinary 20-methylsteroids may arise directly from related 20-methylsteroids present in the adrenal glands, but there is good reason to suppose that they may in part arise by metabolic reduction at C-21 of 21-hydroxysteroids secreted by the glands. The evidence that such metabolic reduction of 21-hydroxysteroids to 20-methylsteroids, i.e. to 21-deoxysteroids can occur in the human body appears to be conclusive. Thus Cuyler, Ashley & Hamblen (1940) and Horwitt, Dorfman, Shipley & Fish (1944) isolated pregnane-3α:20α-diol from the urine of human subjects after the administration of 11-deoxycorticosterone acetate; Mason (1948) isolated pregnane-3α:20α-diol-11-one after administration of 11-dehydrocorticosterone; while more recently Lieberman, Hariton, Stokem, Studer & Dobriner (1951) have isolated pregnane-3α:17α:diol-11:20-dione after administration of cortisone acetate. It has been suggested that the principle C_{21} adrenocortical hormone secreted by the adrenal glands in man may be 17-hydroxycorticosterone (Reich, Nelson & Zaffaroni, 1950; Hechter, 1950; Pincus, Hechter & Zaffaroni, 1951). In view of this possibility and /

and because of the widespread therapeutic use of 17-hydroxy-11-dehydrocorticosterone (cortisone), it was thought that a specific method for the quantitative determination in urine of C_{21} 17:20-dihydroxy-20-methylsteroids which might be formed from these two hormones (and from 17-hydroxy-11-deoxycorticosterone) by metabolic reduction of the C-21 hydroxyl group, would be of some value and interest.

C_{21} steroids with either α-ketol or α-glycol side chains give almost quantitative yields of formaldehyde on oxidation with periodic acid, and this reaction has been widely employed for the quantitative determination in suitably prepared urine extracts of the so-called 'formaldehydogenic' steroids (1,2). On oxidation with periodic acid, C_{21} 17:20-dihydroxy-20-methylsteroids, and steroids of no other type, should yield acetaldehyde. Accordingly, the possibility was envisaged of determining both formaldehydogenic and acetaldehydogenic steroids in urinary extracts by periodate oxidation with separation and estimation of the formaldehyde and acetaldehyde thus produced. Talbot & Eitingon (1944) have estimated the 17-ketosteroids formed after periodate oxidation of urine extracts, thus measuring the combined total of C_{21} 17:20:21-trihydroxysteroids, C_{21} 17:20-dihydroxy-20-methylsteroids and C_{21} 17-hydroxy-20-keto-20-methylsteroids (cf. Fig. 1, Section 1, 2).

With pregnane-3α:17α:20α-triol (Butler & Marrian, 1937, 1938) preliminary experiments showed that nearly quantitative /

quantitative yields of acetaldehyde as determined colorimetrically in sulphuric acid with 4-hydroxydiphenyl (Miller & Muntz, 1938; Koenemann, 1940; Barker & Summerson, 1941) could be obtained after oxidation with periodic acid. In further experiments in which mixtures of pregnane-3α:17α:20α-triol and 11-deoxycorticosterone or 17-hydroxy-11-deoxycorticosterone were oxidized with periodic acid, it was found possible to remove the acetaldehyde quantitatively from a reaction mixture containing glycine without loss of formaldehyde, by aeration into a bisulphite trap (cf. Shinn & Nicolet, 1941). The formaldehyde could then be recovered quantitatively in the usual way by distillation (Daughaday et al., 1948).

To test out the potentialities of the procedure for the determination of acetaldehydogenic steroids in urine, recovery experiments were carried out in which known amounts of pregnane-3α:17α:20α-triol were added to both unwashed and sodium hydroxide-washed chloroform extracts of urine from normal men. These extracts were obtained from fresh urine, from urine after incubation with ox-spleen β-glucuronidase at pH 4.5, and from urine extracted after standing at room temperature at pH 1 for 24 hr. In every experiment blank determinations were carried out on a portion of the extract with no added pregnanetriol.

Reasonably satisfactory recoveries were obtained in those experiments in which the triol was added to sodium hydroxide-washed chloroform extracts of urine. In those in which unwashed extracts were employed, the recoveries were irregular /

irregular, indicating possibly the presence of material interfering in some way with the periodate oxidation of the triol. It may be noted that Hollander, Di Mauro & Pearson (1951), studying the recovery in periodate oxidations of 11-deoxycorticosterone added to urine extracts, concluded that the oxidation could be inhibited by substances present in crude extracts.

The 'blank' experiments all indicated the presence of acetaldehydogenic substances in the extracts, and it is noteworthy that from the various extracts the amounts of acetaldehyde obtained were roughly of the same order as those of formaldehyde, and like the latter, were increased both by glucuronidase and acid hydrolysis of the urine (see Section 4). Talbot & Eitingon (1944) when measuring 17:20-dihydroxysteroids in butanol extracts of urine from patients with adrenocortical virilism, found that heating the extracted material at pH 6 or incubation with rat liver enzyme preparations increased the amount of such free steroids; heating with 15% (v/v) HCl caused complete destruction.

It is pertinent to note that Mason & Kepler (1945) isolated from adrenal carcinoma urine a mixture of steroid conjugates which yielded pregnane-3α:17α:20α-triol after incubation with a crude rat liver glucuronidase preparation.

As far as can be judged from recovery experiments with pregnanetriol, the procedure developed should provide a satisfactory method for determining acetaldehydogenic steroids in sodium hydroxide-washed chloroform extracts of urine, and of urine hydrolyzed /

hydrolyzed enzymically or with acid. It is not claimed that this procedure provides a means for the quantitative determination of such steroids in urine, since no demonstrably quantitative methods have yet been developed for the hydrolysis of the conjugated acetaldehydogenic steroids which must be assumed to be present in urine, or for the extraction of the free steroids after hydrolysis. In fact, in these respects the method is neither better nor worse than the widely employed procedures for the determination of urinary reducing or formaldehydogenic steroids, the difficulties involved in which have recently been summarized by Marrian (1951).

EXPERIMENTAL

3, 2. Preliminary Experiments with Acetaldehyde and Formaldehyde.

It was desired to oxidize C_{21} 17:20-dihydroxy-20-methyl-steroids with periodic acid and estimate the acetaldehyde formed; also, if possible, to oxidize simultaneously formaldehydogenic steroids and estimate the formaldehyde liberated. First, the estimation of acetaldehyde and formaldehyde and their separation were investigated.

(a) Estimation of acetaldehyde. For the estimation of 0-5 µg./ml. acetaldehyde the method of Miller & Muntz (1938) as modified by Koeneman (1940) and Barker & Summerson (1941) has been used. This method was originally developed for the estimation of lactic acid in biological material and involves oxidation of lactic acid to acetaldehyde by hot sulphuric acid; the acetaldehyde in the sulphuric acid is estimated by means of the violet colour developed when the solution is treated with 4-hydroxydiphenyl (Eegriwe, 1933).

The author found it necessary to standardize this colorimetric estimation rigorously in order to obtain reproducible results. The method finally adopted is as follows (lithium lactate or paraldehyde are suitable as standards): a 1.0 ml. portion of an aqueous solution containing the equivalent of 0-5 µg. acetaldehyde is transferred to a dry test-tube (65 x 150 mm.) and 7.0 ml. concentrated sulphuric acid A.R. is added from a burette. During this addition the test-tube is kept in an ice-bath and continuously /

continuously agitated[x]. The solution is cooled for 10 minutes in an ice bath and 0.05 ml. 4% (w/v) $CuSO_4,5H_2O$ A.R. is added, the solution is mixed by shaking it, and 0.10 ml. 1.5% (w/v) 4-hydroxydiphenyl in 0.5% (w/v) NaOH is added and dispersed throughout the solution. During colour development the mixture is placed in a thermostatically controlled water bath at 25°. After about 30 min. the sample is mixed again by shaking, and at the end of 60 min. in the water bath, is withdrawn. To destroy excess 4-hydroxydiphenyl the mixture is heated in a boiling water bath for 1.5 min. A glass rod is used to stir the solution during this heating. After cooling to room temperature, the density of the solution is estimated on a Spekker photoelectric absorptiometer using Ilford No. 605 filters, set at zero density with a reagent blank. The coloured solutions are stable for several hours.

The temperature at which colour development is allowed to proceed affects the colour density markedly. Hence, strict adherence to a standard procedure for cooling the sulphuric acid mixtures and a constant temperature bath for colour development are essential.

Particular attention should be paid to the quality of the /

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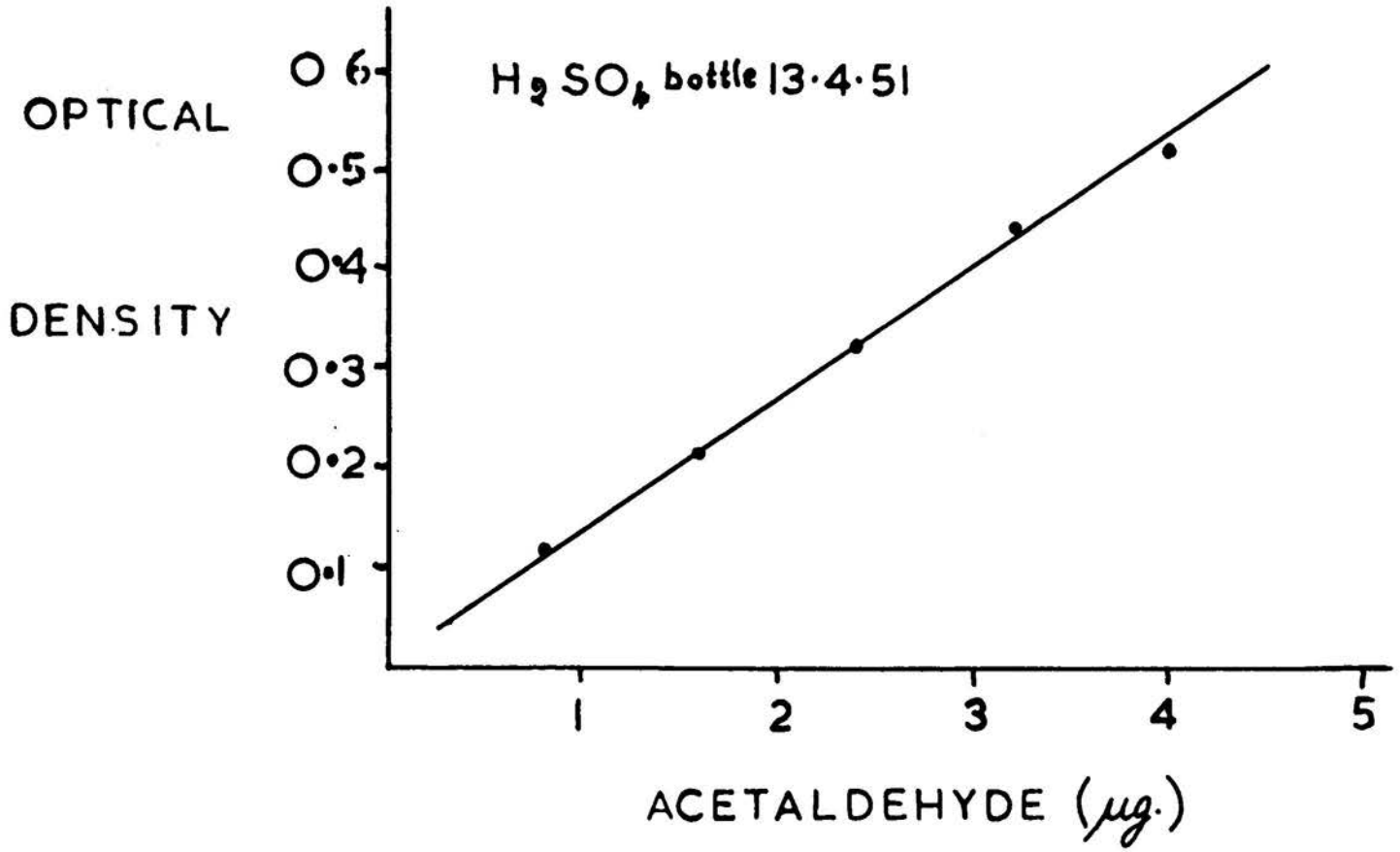
[x] If lactic acid is used as a standard, it must be oxidized to acetaldehyde by heating the acid solution in a boiling water bath for 10 min. before proceeding to the next stage.

FIG. 3. CALIBRATION CURVE USING PARALDEHYDE, BARKER & SUMMERSON (1941) REACTION



the A.R. sulphuric acid. For instance, Russell (1944) has shown that presence of nitrates or nitrites in the sulphuric acid affects the colour development. Each bottle of sulphuric acid should be checked before use and those giving poor colour development discarded. Fresh calibration curves must be prepared with each batch of sulphuric acid (Fig. 3) as considerable variations may be found in the slope of the curve from batch to batch.

Also to be noted is the advice of Barker and Summerson (1941) against the use of chromic acid mixtures for cleaning apparatus used in the acetaldehyde estimations, because of the difficulty of removing interfering traces of the cleaning agent. Rinsing the apparatus with ethanol prior to final distilled water washing overcomes this difficulty.

(b) Estimation of formaldehyde. The quantitative colorimetric estimation of formaldehyde using chromotropic acid [x] in sulphuric acid was described by McFadyen (1945). This method was used by Corcoran et al. (1948) and Daughaday et al. (1948) for the estimation of formaldehydogenic corticoids in urine. Its accuracy and reproducibility were checked by Dr. Paterson (1950) in this laboratory.

An aqueous solution (3.0 ml.) containing 0 - 15 µg. formaldehyde is mixed with 5.0 ml. 0.2% chromotropic acid in 30 N-H_2SO_4 (A.R.) in a test-tube and the mixture heated for 30 min. in a boiling water bath. After cooling the mixture for 5 min. in a cold water bath, the volume is made up to 10.0 ml. with/

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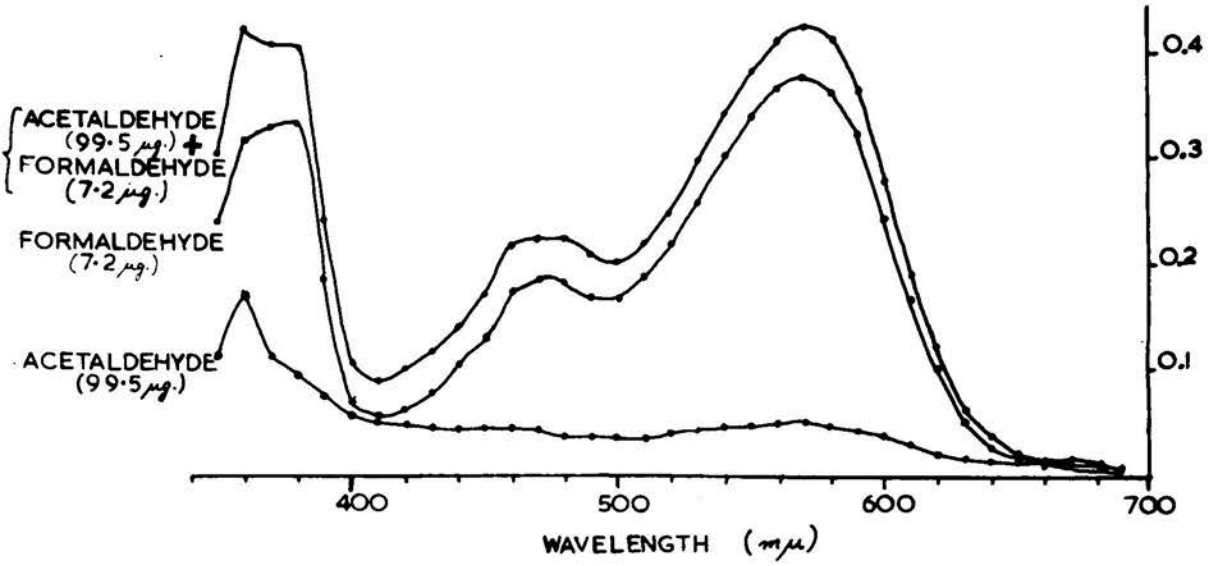
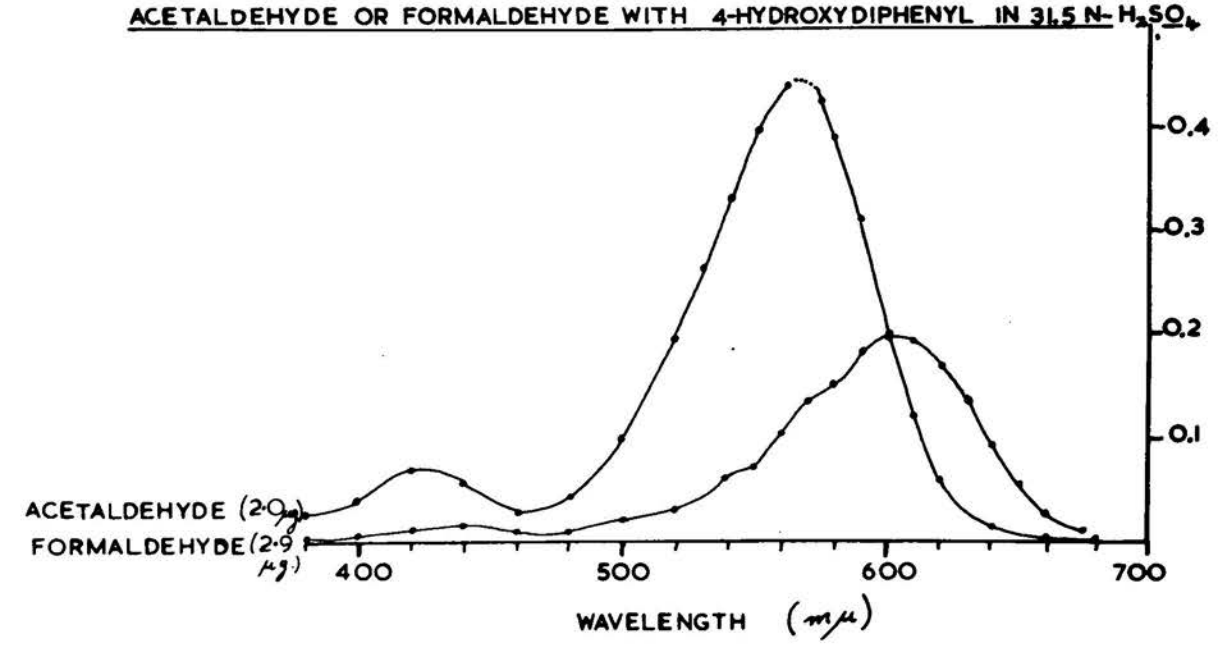
[x] chromotropic acid - 1:8-dihydroxy-naphthalene-3:6-disulphonic acid

FIG. 4. ABSORPTION SPECTRA OF SOLUTIONS AFTER REACTION OF ACETALDEHYDE OR FORMALDEHYDE WITH CHROMOTROPIC ACID IN 18N-H_2SO_4



ABSORPTION SPECTRA OF SOLUTIONS AFTER REACTION OF ACETALDEHYDE OR FORMALDEHYDE WITH 4-HYDROXYDIPHENYL IN 31.5 N-H_2SO_4



with 18 N-H_2SO_4 (A.R.) and the solution thoroughly mixed with a glass stirrer. Similarly, a reagent blank is prepared. The density of the solutions is determined with a Spekker photo-electric absorptiometer using Ilford No. 605 filters, set at zero density for the reagent blank.

(c) Absorption spectra of solutions after reaction of acetaldehyde and formaldehyde with chromotropic acid and 4-hydroxydiphenyl. The colour reactions of the aldehydes with chromotropic acid and 4-hydroxydiphenyl in H_2SO_4 were described by Wegriwe (1933). After reaction of the aldehydes with 4-hydroxydiphenyl and with chromotropic acid (as above) the absorption spectra of the coloured complexes was measured between 340 mμ and 700 mμ on a Unicam S.P. 500 photoelectric quartz spectrophotometer (Fig. 4). Reaction with 4-hydroxydiphenyl in sulphuric acid. The maximum density for the formaldehyde complex is 600-610 mμ; for the acetaldehyde complex, 565 mμ. This reaction is suitable for the quantitative estimation of either acetaldehyde or formaldehyde but not for a mixture of the two aldehydes. Reaction with chromotropic acid in sulphuric acid. The maxima for the formaldehyde complex are 570 mμ/480 mμ and 380 mμ. The acetaldehyde complex has a maximum density at 360 mμ and shows general absorption over the range of wavelengths between 400 mμ and 620 mμ. This reaction is not suitable for the estimation of acetaldehyde but it should be noted that presence of acetaldehyde may result in overestimation of formaldehyde.

In/

In neither of these reactions is it possible to estimate acetaldehyde and formaldehyde together; in fact, in either reaction it is necessary to have only one of the aldehydes present for accurate estimation.

(d) Separation of acetaldehyde and formaldehyde. Experiments with very dilute aqueous solutions of acetaldehyde and formaldehyde (containing about 10 µg. of each) showed that acetaldehyde could be removed quantitatively by aeration [x] without loss of formaldehyde in the presence of 1% glycine (cf. Shinn & Nicolet, 1941). The acetaldehyde was trapped in 1% sodium bisulphite solution. In the method of Daughaday et al. (1948) for the estimation of formaldehydogenic steroids, after oxidation with periodic acid formaldehyde is distilled out of the reaction mixture. Both acetaldehyde and formaldehyde were found by the author to be quantitatively distilled, and the aldehydes, in the distillate (which was collected in 1% glycine solution) could be separated by aeration [x].

(e) Oxidation of pregnane-3α:17α:20α-triol. In oxidation for one hour at room temperature acetaldehyde was not obtained quantitatively/

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[x] Where a number of aerations were being carried out the sets of tubes and traps were connected up in parallel to the air supply. Aeration of a train connected up in series gave quantitative recovery of acetaldehyde only in the first set. The cause of this was not investigated. This effect was also observed if concentrated sulphuric acid was used as a trap for acetaldehyde.

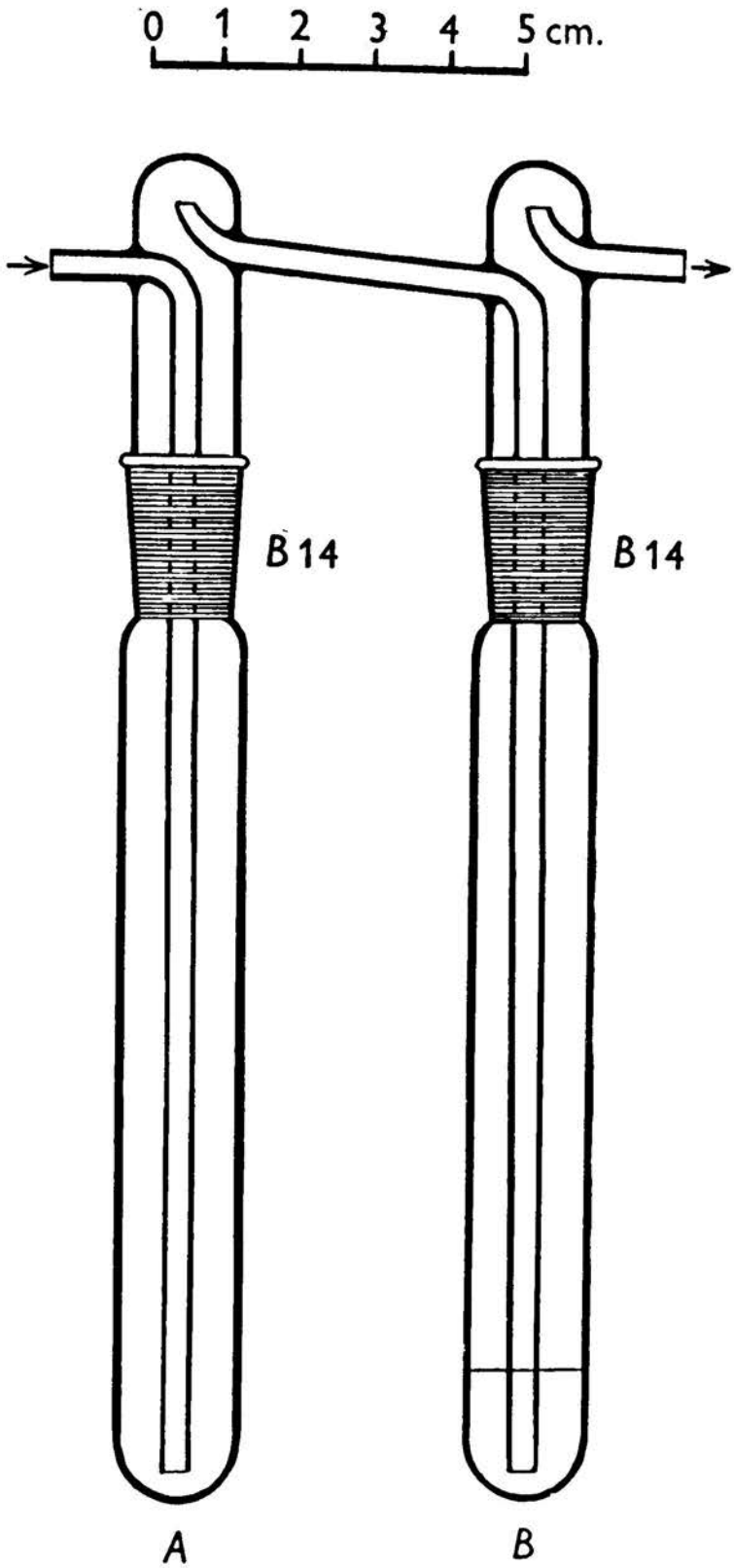


Fig. 5. Apparatus used for oxidation with aeration. *A*, oxidation tube; *B*, bisulphite trap graduated at 3·0 ml. Arrow indicates direction of air stream.

quantitatively from pregnanetriol without using such high concentrations of periodic acid (0.33 M in 0.5 N-H_2SO_4) that great difficulties arose in the distillation procedure. It was found that by aerating acetaldehyde out of the oxidation mixture during oxidation, quantitative yields of the acetaldehyde were obtained in less than 1 hr. at room temperature.

3, 3. Method for the estimation of acetaldehydogenic and formaldehydogenic steroids.

(a) Apparatus. The apparatus used is shown in Fig. 5. A is the oxidation tube; B contains aqueous bisulphite solution in which acetaldehyde, carried over from the oxidation mixture in the air stream, is trapped. A second bisulphite trap has not been found necessary under the conditions used.

Pregnane-3α:17α:20α-triol, m.p. 245-247° (Butler & Marrian, 1938) has been used throughout as the reference acetaldehydogenic steroid.

(b) Method. Ethanolic solutions of pure steroids or of suitably prepared urine extracts are evaporated in oxidation tubes A (Fig. 5) to about 1 ml. under a rapid stream of filtered air on a water bath at about 80°. Evaporation to dryness is carried out in vacuo with warming on a water bath. The residues are moistened with 0.10 ml. glacial acetic acid, aldehyde free. The tubes are warmed slightly in a water bath and rotated to wet any residue adhering to the sides of the tube. Equal volumes of 0.12 M-periodic acid in 0.4 N-H_2SO_4 A.R. and 1% glycine in 0.4 N-H_2SO_4 A.R. are freshly mixed, and 1 ml. portions of the mixture pipetted /

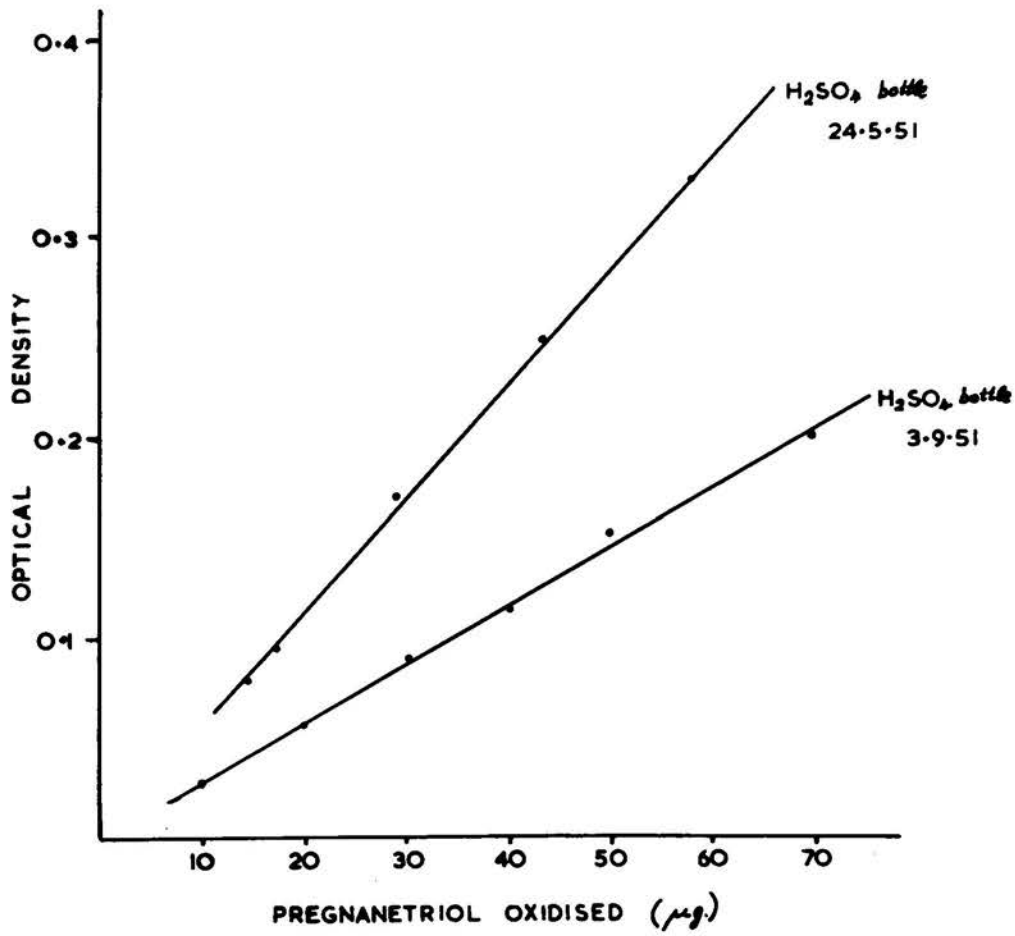
pipetted into the oxidation tubes. The trap tubes B, each containing 2 ml. 1% sodium bisulphite solution are immediately connected up. A slow stream of air, purified by passage through concentrated H_2SO_4 A.R. and a soda lime tower is blown through the sets of tubes arranged in parallel. The rate of aeration is about 5 ml./min.

After 45 min. of oxidation with aeration, the air flow is stopped and the tubes are disconnected. The bisulphite traps are made up to 3.0 ml. (graduation mark) by washing down the ends of the connecting tubes with distilled water, and acetaldehyde is estimated in 1.0 ml. samples of the solutions as described in 3, 2(a).

The formaldehyde retained in the oxidation mixture is estimated by a modification of the method of Daughaday et al. (1948). The oxidation mixture is diluted to about 4 ml. and transferred to a small round-bottomed long-necked distilling flask (bulb capacity about 25 ml.) fitted with a B19 ground-glass joint. The oxidation tube is washed with 2 ml. water and 1.0 ml. aldehyde free ethanol, which are transferred to the distilling flask. The excess periodic acid is reduced with 3.0 ml. 6% (w/v) stannous chloride A.R. in N-HCl, and after the addition of 3.0 ml. 10 N-H_2SO_4 A.R. the mixture is slowly distilled through an all-glass micro-still (Paterson, 1950) into 1.0 ml. 4% (w/v) sodium sulphite A.R. in aqueous 1% (v/v) aldehyde free ethanol. The tip of the condenser is kept immersed in the alcoholic sulphite solution until about 4 ml. of distillate have collected.

The /

FIG. 6. CALIBRATION CURVES: OXIDATION OF PREGNANE-3α:17α:20α-TRIOL WITH PERIODIC ACID & ESTIMATION OF $CH_3.CHO$ FORMED



The condenser tip is then raised above the liquid surface and the distillation continued until a total of 7.0 ml. (graduation mark) is reached. A 3.0 ml. portion is used for the estimation of formaldehyde (3, 2b).

Reagent blanks for the acetaldehyde and formaldehyde estimations are carried through the oxidation, distillation and colour development in the same way as the urine extracts.

3, 4. Optimum conditions for the oxidation of pregnane-3α:17α:20α-triol.

The effect of varying periodic acid concentration, time of aeration and acidity of the oxidation mixture on the yield of acetaldehyde obtained by periodate oxidation of pregnanetriol are illustrated in Tables 4, 5, and 6. Apart from the variable studied, other conditions in these experiments were as described in the method. The oxidation-aerations have been carried out at room temperature, i.e. 13-21° and within this range the recoveries of acetaldehyde have not been affected by temperature.

Table 7 shows the recoveries of acetaldehyde after oxidation of pregnanetriol with 0.06 M-periodic acid in 0.4 N-sulphuric acid with aeration for 45 min. as in the method finally adopted. Since the method gave practically quantitative yields of acetaldehyde from pregnanetriol, calibration curves were prepared directly using pregnanetriol itself as a standard (Fig. 6). The calibration curves were re-determined for each bottle of sulphuric acid used (3, 2a).

TABLE 4. /

TABLE 4. Effect of periodic acid concentration on oxidation of pregnane-3α:17α:20α-triol

(58.0 μg. pregnanetriol samples oxidized)

Concn. of periodic acid (M)	Acetaldehyde found (μg.)	Equivalent amount of triol (μg.)	Recovery (%)
0.24	7.80	59.5	103
	7.80	59.5	103
0.12	7.61	58.0	100
	7.80	59.5	103
0.04	7.87	60.0	103
	6.69	51.0	88
0.01	5.50	42.0	72
	6.69	51.0	88

TABLE 5. Effect of oxidation-aeration time on yield of acetaldehyde from pregnanetriol oxidized with 0.06 M-periodic acid

(25.1 μg. pregnanetriol samples oxidized)

Time aerated (min.)	Acetaldehyde found (μg.)	Equivalent amount of triol (μg.)	Recovery (%)
5	0.11	0.9	4
10	0.45	3.4	13
15	0.62	4.7	19
20	1.13	8.6	34
25	2.29	17.4	69
30	3.07	23.4	93
40	3.32	25.2	100
50	3.27	24.9	99

TABLE 6. /

TABLE 6. Effect of acidity of oxidation mixture on yield of acetaldehyde from pregnane-3α:17α:20α-triol in periodate oxidation

(25.1 μg. samples pregnanetriol oxidized)

Normality of H_2SO_4 in oxidation mixture	Acetaldehyde found (μg.)	Equivalent amount of triol (μg.)	Recovery (%)
0	0.87	6.7	27
0.01	2.31	17.6	70
0.05	2.97	22.8	90
0.1	2.76	21.1	84
0.2	3.21	24.5	98
0.4	3.35	25.6	102
0.8	3.23	24.7	98

TABLE 7. Recovery of acetaldehyde on oxidation of pregnanetriol by the method finally adopted

(Oxidation with 0.06 M-periodic acid in 0.4 N-H_2SO_4)

Pregnanetriol oxidized (μg.)	Acetaldehyde found (μg.)	Equivalent amount of triol (μg.)	Recovery (%)
58.0	7.38	56.3	97
43.5	5.58	42.6	98
29.0	3.82	29.2	101
17.4	2.16	16.5	95
14.5	1.78	13.6	94

3, 5. Oxidation /

TABLE 8. Recovery of acetaldehyde and formaldehyde from periodate 11-deoxycorticosterone (DOC) and

Amount of steroid oxidized (μg.)			Acetaldehyde found (μg.)	Formaldehyde found (μg.)
Triol	DOC	S		
58.0		36.0	7.42	2.76
34.8		36.0	4.50	2.76
11.6		36.0	1.60	3.13
52.7		54.0	6.05	5.04
31.6		54.0	3.96	4.90
21.1		54.0	2.88	4.30
10.5		54.0	1.40	4.57
52.7		72.0	6.66	5.84
31.6		72.0	3.49	5.78
21.1		72.0	2.97	5.73
52.7	63.2		6.33	5.25
31.6	63.2		4.05	5.46
10.5	63.2		1.47	5.73
52.7	63.2		6.75	5.67
31.6	15.8		4.50	1.43
21.1	15.8		2.70	1.48
10.5	15.8		1.35	1.38
52.7	31.6		6.75	2.70
31.6	31.6		3.96	2.65
10.5	31.6		1.37	2.81

3, 5. Oxidation of mixtures of acetaldehydogenic and formaldehydogenic steroids.

Preliminary experiments with aqueous solutions of acetaldehyde and formaldehyde showed that a satisfactory separation of the aldehydes at levels of 10 μg. or less was possible under the conditions used for the oxidation and estimation of pregnanetriol. These conditions would be expected to permit quantitative formation of formaldehyde from formaldehydogenic steroids [x]. It was therefore probable that the acetaldehyde and formaldehyde formed on periodate oxidation of mixtures of C_{21} steroids with various types of C-17 side chains could be separated and hence 10-100 μg. amounts of steroids estimated quantitatively.

From mixtures of pregnane-3α:17α:20α-triol and 11-deoxycorticosterone or 17-hydroxy-11-deoxycorticosterone, the amounts of acetaldehyde and formaldehyde recovered were consistent with the respective amounts of steroids present [x] (Table 8).

3, 6. Oxidation of C_{21} 17-hydroxy-20-keto-20-methylsteroids with periodic acid.

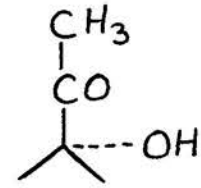
An experiment was carried out to check that a steroid with a 17-hydroxy-20-keto-20-methyl side chain would not give rise to acetaldehyde on oxidation with periodic acid under the conditions /

---

[x] Experiments with 11-deoxycorticosterone or 17-hydroxy-11-deoxycorticosterone alone confirmed that formaldehyde was quantitatively recovered under the conditions used.

conditions used. Such a compound would be expected to give acetic acid and a 17-ketosteroid (see Section 1, 2).

FIG. 7. 17-hydroxy-20-keto-20-methyl side chain



Samples of pregnane-3α:17α-diol-20-one, m.p. 208.5-209.5° (a gift from Dr. Gallagher to Professor Marrian) and pregnanetriol were oxidized with periodic acid as usual. The results (Table 9) indicate that no significant amounts of acetaldehyde are liberated from pregnane-3α:17α-diol-20-one, i.e. from a 17-hydroxy-20-keto-20-methyl side chain. The Spekker readings obtained after oxidation of pregnane-3α:17α-diol-20-one are in fact not reliable at such low levels.

TABLE 9. Oxidation of pregnane-3α:17α-diol-20-one with periodic acid

Steroid oxidized	Amount oxidized (μg.)	Spekker reading	Acetaldehydogenic steroid found (μg.)	Recovery (%)
Pregnane-3α:17α-diol-20-one	34	0.010	1.8	5
	34	0.011	1.9	6
	34	0.009	1.6	5
	34	0.007	1.2	4
Pregnane-3α:17α:20α-triol	85	0.475	83.1	98
	85	0.460	80.5	95

3, 7. Recovery of pregnanetriol added to urine extracts, and to urine. Neutralization of urine prior to extraction. For comparative purposes it is desirable to extract urine samples after various pretreatments under similar conditions. In most experiments reported in this Section, prior to extraction, urine samples were brought /

brought to pH 6.9-7.1 using 2 N-sodium carbonate and 0.5 M-K_2HPO_4. The samples were stirred vigorously during the addition of alkali.

The recoveries of pregnanetriol added to chloroform extracts of unhydrolyzed, acid and enzymically hydrolyzed urine were studied, in addition to the recovery of pregnanetriol added to urine itself (added in 0.5-1.0 ml. ethanol) with subsequent extraction of the urine and purification of extracts. Recoveries were calculated from the increase in acetaldehyde obtained from extracts containing added pregnanetriol over that given by extracts without added triol.

The recoveries of pregnanetriol added to chloroform extracts of urine where no alkali washing was carried out, varied from urine to urine (Table 10).

TABLE 10. Irregular recovery of pregnanetriol added to unwashed chloroform extracts of normal male urine

		Pretreatment of urine					
		(1) None		(2) Acid hydrolysis		(3) Enzymic hydrolysis	
	Triol added (µg.)	Triol recovered (%)	Urine 'blank' (mg. as triol/ 24 hr.)	Triol recovered (%)	Urine 'blank' (mg. as triol/ 24 hr.)	Triol recovered (%)	Urine 'blank' (mg. as triol/ 24 hr.)
Urine I	0		0.98		2.15		3.90
	0		0.97		2.02		4.12
	10	19		102		35	
	25	8		54		69	
	50	62		52		59	
Urine	0		0.86		2.63		3.64
	0		0.94		2.79		3.88
	10	82		95		117	
	25	103		101		90	
	50	100		93		77	

With /

With alkali-washed extracts, recoveries were consistent, and are illustrated in the results obtained for the recovery of pregnanetriol added to urine itself with subsequent chloroform extraction (Table 11).

TABLE 11. Recovery of pregnanetriol added to urine from normal male subjects. Chloroform extracts alkali-washed.

(31.5 µg. triol added to each sample)

Subject	Urine 'blank' value (as mg. triol/day)	Recovery of added triol (%)
G.W.	0.33	96
	(0.26)[x]	-
A.K.L.	0.48	94
	0.49	95
W.T.	0.40	93
	0.40	93
R.I.C.	0.26	90
	0.29	93

[x] Loss during processing

3, 8. Acetaldehydogenic substances in urine.

The urine 'blank' values shown in Tables 10 and 11, i.e. acetaldehyde obtained from urine extracts without added pregnanetriol, are a measure of the acetaldehydogenic substances extractable from urine and may indicate the presence of C_{21} 17:20-dihydroxy-20-methylsteroids. The 'blank' values given in Tables 10 and 11 are expressed as mg. pregnanetriol/24 hr.

In Table 12 are compared 'blank' values obtained from untreated urine, acid hydrolyzed and enzymically hydrolyzed urine from normal male subjects. The values obtained after acidification /

TABLE 12. Amounts of acetaldehydogenic material found in urine of normal male subjects

(All values expressed as mg. pregnanetriol / 24 hr.)

Urine sample	Untreated urine	Acidification to pH 1; immediate extraction	Acidification to pH 1; extracted after 24 hr.	Incubated at 37°, pH 4.5, with β-glucuronidase
	Treatment of urine prior to chloroform extraction			
	A. Chloroform extracts alkali-washed			
I	0.33	0.44	0.92	-
II	0.28	0.43	-	-
III	0.36	-	-	1.58
IV	0.47	-	-	-
V	0.48	0.93	0.80	1.94
VI	0.12	0.19	0.29	0.66
VII	0.28	0.32	0.45	-
	B. Chloroform extracts not alkali-washed			
VIII	0.92	2.96	2.80	3.60
IX	0.72	3.20	3.40	4.80

acidification or incubation with β-glucuronidase preparations are considerably higher than those from untreated urine. Similar effects have been observed for urinary formaldehydogenic substances; the hydrolysis of urinary conjugated acetaldehydogenic and formaldehydogenic substances is discussed in Section 4.

3, 9. DISCUSSION

Estimation of acetaldehyde formed by periodate oxidation of urine extracts is more specific and sensitive than the estimation of 17-ketosteroids formed (Talbot & Eitingon, 1944). As well as C_{21} 17:20-dihydroxy-20-methylsteroids, 17:20:21-trihydroxy- and 17-hydroxy-20-keto-20-methyl-steroids yield 17-ketosteroids on periodate oxidation, and thus these types of steroids are estimated together in the latter method. It is desirable for metabolic studies that these steroids, which include both 21-hydroxy- and 21-deoxy-steroids, should be estimated separately. In addition to the general disadvantages of the 17-ketosteroid methods, the Talbot & Eitingon (1944) method estimates the relatively small increase of 17-ketosteroids after periodate oxidation over that present before oxidation.

It has been found that neutral chloroform extracts of urine from normal men contain substances which liberate acetaldehyde when oxidized with periodic acid under the same conditions necessary for quantitative oxidation of 17:20-dihydroxy-20-methylsteroids. Although such steroids have been isolated

from urine of patients with hyperactive adrenal glands, proof of the presence of such compounds in the urine of normal subjects must await isolation studies. Preliminary experiments in such studies have shown that pregnane-3α:17α:20α-triol occurs in the urine of normal men (Section 6).

The presence of some types of conjugates was indicated by the increased amounts of acetaldehydogenic material extractable from urine after treatment with acid or β-glucuronidase preparations. Relatively mild hydrolysis conditions were employed to avoid excessive destruction of the sensitive C-17 side chain (in particular the tertiary hydroxyl at C-17). Hydrolysis of urinary conjugates is considered in more detail in Section 4.

Acetaldehyde gives a brown coloration with chromotropic acid and may interfere in the estimation of formaldehyde if present in relative excess over the latter (McFadyen, 1945). Where estimation of the acetaldehyde is not desired, it may be removed by aeration from the formaldehyde distillates in the Daughaday (1948) method if 1% aqueous glycine is used in place of the ethanolic sulphite solution.

SUMMARY /

SUMMARY

1. A method for estimating C_{21} 17:20-dihydroxy-20-methylsteroids in urine, based on periodic acid oxidation, is described.

2. The method allows of the simultaneous estimation of acetaldehydogenic and formaldehydogenic steroids.

3. A urinary excretion of unconjugated acetaldehydogenic substances equivalent to 0.1-0.5 mg./day of C_{21} 17:20-dihydroxy-20-methylsteroids was found for seven normal men.

4. The presence of conjugated acetaldehydogenic substances in normal men's urine is also indicated.

SECTION 4.

SOME PRELIMINARY EXPERIMENTS ON THE HYDROLYSIS OF URINARY CONJUGATED FORMALDEHYDOGENIC AND ACETALDEHYDOGENIC SUBSTANCES.

4, 1. INTRODUCTION.

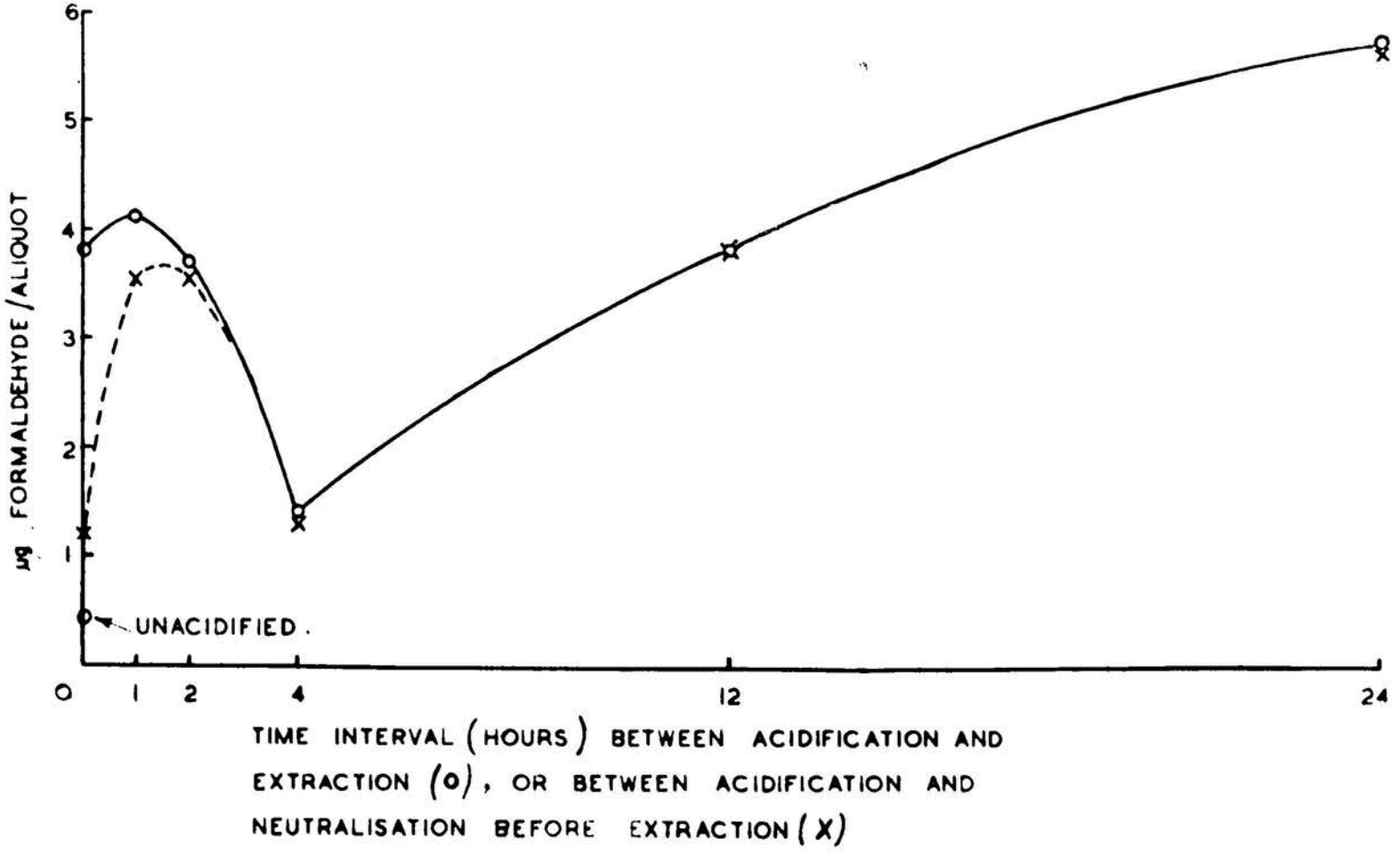
No conjugates of formaldehydogenic steroids have been isolated from urine yet. In the case of acetaldehydogenic steroids, Mason & Kepler (1945) isolated what appeared to be a mixture of steroid glucuronides including that of pregnane-3α:17α:20α-triol from the urine of a patient with adrenal hyperplasia. The problem of the hydrolysis of possible conjugates of such steroids in urine was studied by observing the conditions necessary for optimum yield of AS and FS; acid and enzymic hydrolyses were investigated.

Until the method described in Section 3 was developed, only conjugated FS were studied using a method of estimation based on that of Daughaday et al. (1947, 1948); thereafter, conjugates of both AS and FS were investigated using the method given in Section 3. Accordingly work in this Section is divided into two sub-sections, A and B; sub-section A deals only with formaldehydogenic substances.

A. Formaldehydogenic Substances.

The author co-operated with Dr. J.Y.F.Paterson in studying the effect of time on the acid hydrolysis of urinary conjugated FS. Dr. Paterson had found (Paterson, 1950; Paterson, Cox & Marrian, 1950) that the amounts of FS extracted by chloroform/

FIG. 8.


6
5
4
3
2
1
µg. FORMALDEHYDE/ALIQUOT
UNACIDIFIED.
0
1
2
4
12
24
TIME INTERVAL (HOURS) BETWEEN ACIDIFICATION AND EXTRACTION (O), OR BETWEEN ACIDIFICATION AND NEUTRALISATION BEFORE EXTRACTION (X)

chloroform varied with the time the urine had been at pH 1 (Fig. 8, circles). If the urine samples were neutralized immediately before extraction the values which were obtained are shown by crosses in Fig. 8. These results were explained by postulating two distinct types of acid-hydrolyzable conjugated FS and the following working hypothesis has been adopted. In one group, the FS is conjugated with some acidic substance. At pH 1 the ionisation of the conjugate is strongly repressed and the unionised form is extractable from the acidified urine by chloroform (chloroform soluble conjugated substance, CSCS). When the urine is neutral the conjugate is ionised and the ionised form is not chloroform extractable. The diminishing effect of neutralization with time indicates a rapid hydrolysis of the conjugate, the hydrolysis being complete within about 2 hr. at pH 1, 25° in urine. The subsequent fall between 2 and 4 hr. in the amount of FS extracted demonstrates the destruction of the free FS liberated by acid hydrolysis. The slow rise after 4 hr. in the amount of FS extracted by chloroform is due to the slower liberation of FS from a conjugate which is not extractable from chloroform at pH 1 (chloroform insoluble conjugated substance, CICS). Extraction at pH 7 and pH 1 gives identical results. The material extracted by chloroform from fresh untreated urine is taken to be free FS.

Most urine specimens give acid hydrolysis curves of the type shown in Fig. 8. Some, however, give different acid hydrolysis /

hydrolysis curves which appear to be due to the relatively small amount or absence of one or other of the types of conjugate (Paterson, 1950).

The CSCS and free FS can be separated from CICS easily, by extracting urine rapidly immediately after acidification (Paterson & Marrian, 1951; Venning, 1952; Bayliss, 1952). Thus the two types of conjugate can be studied separately; preliminary studies on the properties of the CICS are reported in this Section. Both acid hydrolysis and hydrolysis with β-glucuronidase preparations have been investigated.

It was found that the CICS could be extracted from urine by n-butanol and that such extracts, or urine itself, on incubation with preparations of ox-spleen β-glucuronidase yielded much larger amounts of FS than could be obtained by acid hydrolysis.

B. Formaldehydogenic and Acetaldehydogenic Substances.

The effects of pH and time on the enzymic hydrolysis have been studied; the pH optimum for the release of both FS and AS from conjugates was found to be pH 4.5, and the rate of hydrolysis was greatest in the first few hours, but relatively slow after 24 hr. Effective inhibition (91 - 102%) of the enzymic hydrolysis by D-glucosaccharic acid (Karunairatnam & Levvy, 1949) was a strong indication that β-glucuronidase was the enzyme involved.

Although the extraction of conjugated FS and AS from urine/

urine with n-butanol may not be complete even in 3 extractions with equal volumes, enzymic hydrolysis appears to be more efficient when carried out on butanol-extracted material. Considerable amounts of conjugated FS and AS were removed if n-butanol solutions of the conjugates were washed with 0.1 N-NaOH; washing with salt saturated 1% Na_2CO_3 resulted in much smaller losses. Although no methods have been developed for the quantitative (as distinct from optimum) hydrolysis of urinary conjugates, it is hoped that the data which have been collected will be a foundation for further work leading to quantitative methods.

4,2. METHODS USED.

The method of Daughaday et al. (1948) as modified by Paterson (1950) was used for the estimation of FS in sub-section A.

After the development of a method for estimating acetaldehydogenic steroids (Section 3) FS and AS were estimated together (sub-section B).

Sub-section A. Estimation of urinary formaldehydogenic substances.

Duplicate portions of urine were shaken in separating funnels successively with 0.875, 0.875 and 0.25 vol. chloroform. Each shaking was continued for 3 min. and centrifugation (1,700 r.p.m. for 10 min.) used, if necessary, to separate the phases. The combined chloroform extracts from each urine sample were dried for 20 min. with 5 g. anhydrous sodium sulphate A.R. The sodium sulphate was filtered off and washed twice with 15 ml. dry chloroform. The chloroform extracts and washings were distilled to dryness under reduced pressure in a water bath at under 60°. Each residue was warmed gently with 10 ml. benzene and the mixture allowed to stand for 30 min. at room temperature. The benzene was filtered into a small round-bottomed long-necked distilling flask (bulb capacity about 25 ml.) fitted with a standard B19 ground glass joint. The residue was washed with 5 ml. benzene and the benzene extract and washing taken to dryness in the distilling flasks under reduced pressure at 60°. The residue was dissolved in 1.0 ml. aldehyde free ethanol and 2.0 ml. 0.01 M-periodic acid in 0.30 N-H_2SO_4 A.R. added. The

flask/

flask was stoppered and allowed to stand for 45 min. at 25°. After adding 5.0 ml. distilled water the oxidation was stopped by the addition of 1.0 ml. 6% (w/v) stannous chloride A.R. in N-HCl. To the mixture was added 3 ml. 10 N-H_2SO_4 A.R., and formaldehyde was distilled out of the mixture through an all glass micro-still (Paterson, 1950) and estimated as described in Section 3,2b.

Preparation of β-glucuronidase concentrates.

For the author's initial experiments with the enzyme Dr. E.S.Sutherland kindly supplied β-glucuronidase solutions. These were prepared (Sutherland, 1950) according to the following outline. The dry residue after acetone extraction of minced spleen was extracted with water, steeped in acetate buffer pH 5.0 for 6 hr. at 25° and centrifuged. The crude enzyme was precipitated from the supernatant made 50% saturated with respect to ammonium sulphate. After dialysis the preparation was further purified by rejecting the precipitate formed when the solution was made 21% saturated with respect to ammonium sulphate. Dialysis to remove the ammonium sulphate gave the enzyme preparation as used.

The method of preparation and assay of β-glucuronidase used in all other experiments has been described in Section 2,5.

EXPERIMENTAL

(A) Formaldehydogenic Substances.

4,3. Acid hydrolysis of conjugated formaldehydogenic substances.

This experiment was typical of many carried out by Dr. J.Y.F.Paterson and the author to obtain data on 'hydrolysis curves'.

A 24 hr. urine specimen (G.S.B., volume 1660 ml.) was diluted to 3000 ml., and a 200 ml. sample withdrawn for assay. The remainder of the urine was acidified to pH 1.0, and allowed to stand at 25°. Duplicate 200 ml. aliquots were withdrawn at various time intervals. One sample of each pair was neutralized to pH 7.0 before chloroform extraction; the other was extracted at pH 1. The formaldehydogenic material extractable from these samples and from the untreated urine was estimated. The results are shown in Table 13 (cf. Fig. 8, Section 4,1).

TABLE 13. Amount of FS extractable from urine at pH 1, 25° after various time intervals.

Time at pH 1 (hr.)	pH of urine when extracted	Spekker reading	Formaldehyde per aliquot (µg.)
Unacidified urine	-	0.004	0.5
0 (immediate extraction)	1	0.032	3.9
	7	0.009	1.1
1	1	0.034	4.2
	7	0.029	3.6
2	1	0.031	3.8
	7	0.029	3.5
4	1	0.012	1.5
	7	0.011	1.4
12	1	0.032	3.9
	7	0.032	3.9
24	1	0.047	5.8
	7	0.045	5.5

The interpretation of these results has been discussed

in/

in the Introduction, and it was concluded that there are two types of conjugated FS, one extractable from acidified urine by chloroform, the other not.

4,4. n-Butanol extraction of the conjugates.

(a) Extraction of acid hydrolyzable chloroform insoluble conjugated substances (CICS-A) from urine by n-butanol.

By analogy with the known partitions of other steroid conjugates it was expected that the CICS would be removed from urine by n-butanol. To avoid possible destruction of urinary conjugates by prolonged heating, evaporation of the butanol extracts was not attempted. Instead, the amount of CICS-A remaining in the urine was estimated and the amount extracted by the n-butanol was obtained by difference.

400 ml. urine (RIC) was extracted, after acidification to pH 1.0, with chloroform (3 x 1.5 vol.) to remove CSCS and free steroids. One half of this chloroform-extracted urine was retained as 'control' sample whereas the other half was extracted with n-butanol (3 x 0.25 vol.). The excess butanol was extracted from the urine with ether (3 x 0.25 vol.). The urine samples were allowed to hydrolyze for 24 hr. at 25° before they were extracted with chloroform and the free FS estimated (Urine I).

A second experiment using pooled urine was carried out in exactly the same way (Urine II).

Results:/

Results:

TABLE 14. Effect of n-butanol extraction on the CICS-A in urine.

Urine sample	Treatment	Spekker reading	Formaldehyde per sample (µg.)
I	control	0.049	2.2
	(no butanol extraction)	0.052	2.3
	extracted with butanol	0.031	1.4
		0.030	1.3
II	control	0.040	1.8
	(no butanol extraction)	0.034	1.5
	extracted with butanol	0.026	1.2
		0.028	1.2

The n-butanol-ether extractions at pH 1 appear to have removed 30-40% of the urinary CICS-A.

(b) Effect of pH on the n-butanol extraction of CICS-A from urine.

Pooled urine from normal male subjects was used. 1,000 ml. urine, after acidification to pH 1.0, was extracted with chloroform (3 x 1.5 vol.). Three portions equivalent to 200 ml. urine were then each extracted with butanol (3 x 0.25 vol.), and with ether (3 x 0.25 vol.) at pH 1.0, pH 7.0, and pH 12.0 respectively. A fourth portion was extracted at pH 1.0 with butanol (6 x 0.25 vol.) and with ether (3 x 0.25 vol.). The fifth portion was not extracted with butanol or ether and was retained as a control. The portions extracted with butanol and ether at pH 7.0 and pH 12.0 were re-acidified to pH 1.0. All the/

the acidified urines were allowed to stand 15 hr. at 25° before chloroform extraction and estimation of FS.

Results.

TABLE 15. Effect of pH on the n-butanol extraction of CICS-A from urine.

pH at which urine was extracted with butanol	Number of 0.25 vol. butanol extractions	Spekker reading	Formaldehyde per sample (µg.)	CICS-A extracted (%)
-	no extraction	0.075	3.31 [x]	-
-	no extraction	0.076	3.36 [x]	-
1	3	0.064	2.83	15
1	3	0.050	2.21	34
7	3	0.058	2.56	23
7	3	0.058	2.56	23
12	3	0.058	2.56	23
12	3	0.060	2.65	21
1	6	0.032	1.41	58
1	6	0.035	1.55	54

[x] average 3.34 used in calculating '% CICS-A extracted'.

There appears to be no significant difference in the amount of CICS-A extractable by butanol from urine at pH 1, pH 7, and pH 12.

Increasing the number of butanol extractions increases the amount of CICS-A extracted, but even after six extractions with 0.25 vol. butanol, at pH 1, only about 56% appears to have been extracted from the acidified urine.

(c) The conjugated corticoid material in n-butanol extracts of urine.

The previous experiments showed that butanol extraction decreased/

decreased the amount of CICS-A in urine. It was desirable to show directly that CICS-A had been extracted by the n-butanol. To avoid distilling the butanol to dryness an attempt was made to re-extract the CICS-A from the butanol with 0.1 N-NaOH.

1000 ml. pooled urine and a 24 hr. specimen (RIC) 1200 ml., collected over 50 ml. butanol were treated separately as follows. Without adjustment of the pH the urine samples were extracted with butanol (6 x 0.33 vol.). The butanol extracts were shaken with 0.1 N-NaOH (3 x 0.1 vol.). To remove excess butanol from the alkali solutions and to remove any free steroids they were washed with ether (4 x 0.25 vol.) and with benzene (2 x 0.25 vol.). With the minimum delay, portions of the alkali solutions were acidified to pH 1.0. The remainder of the alkali solutions was neutralized to pH 7 and stored in the refrigerator.

In the case of the extract from the 24 hr. specimen, a portion of the acidified solution was extracted immediately after acidification with chloroform (6 x 0.1 vol.). The chloroform extract was washed with 0.1 N-NaOH (3 x 0.1 vol.) and with water (3 x 0.1 vol.). The amount of PS in the chloroform extract was then estimated.

The remainder of this acidified extract and the acidified extract from the pooled urine specimen were allowed to stand 24 hr. at 25° before extraction as before.

Results. /

Results.

TABLE 16. CICS-A in n-butanol extracts of urine

Urine sample	Time at pH 1 (hr.)	Formaldehydogenic steroid (mg./l.)
I. Pooled urine	24	0.12
	24	0.12
II. 24 hr. specimen	0	0.05
	0	0.06
	24	0.20
	24	0.17

The results indicate that some of the CICS-A in urine can be extracted with butanol, washed out of the butanol by 0.1 N-NaOH and hydrolyzed by acid.

4,5. Experiments with β-glucuronidase preparations.

(a) Incubation of butanol extracted substances with β-glucuronidase.

It seemed possible that some of the FS of urine might be conjugated with glucuronic acid and form part of the CICS. The effect of incubation of a portion of the extract prepared in the previous experiment (Urine Sample II) with a β-glucuronidase preparation [x] was investigated.

A sample of the extract equivalent to 120 ml. urine was taken to pH 5 with acetic acid and incubated with β-glucuronidase (2,5) for 24 hr. at 37°. The amount of FS obtained was equivalent to 2.0 mg. steroid/litre. This remarkably /

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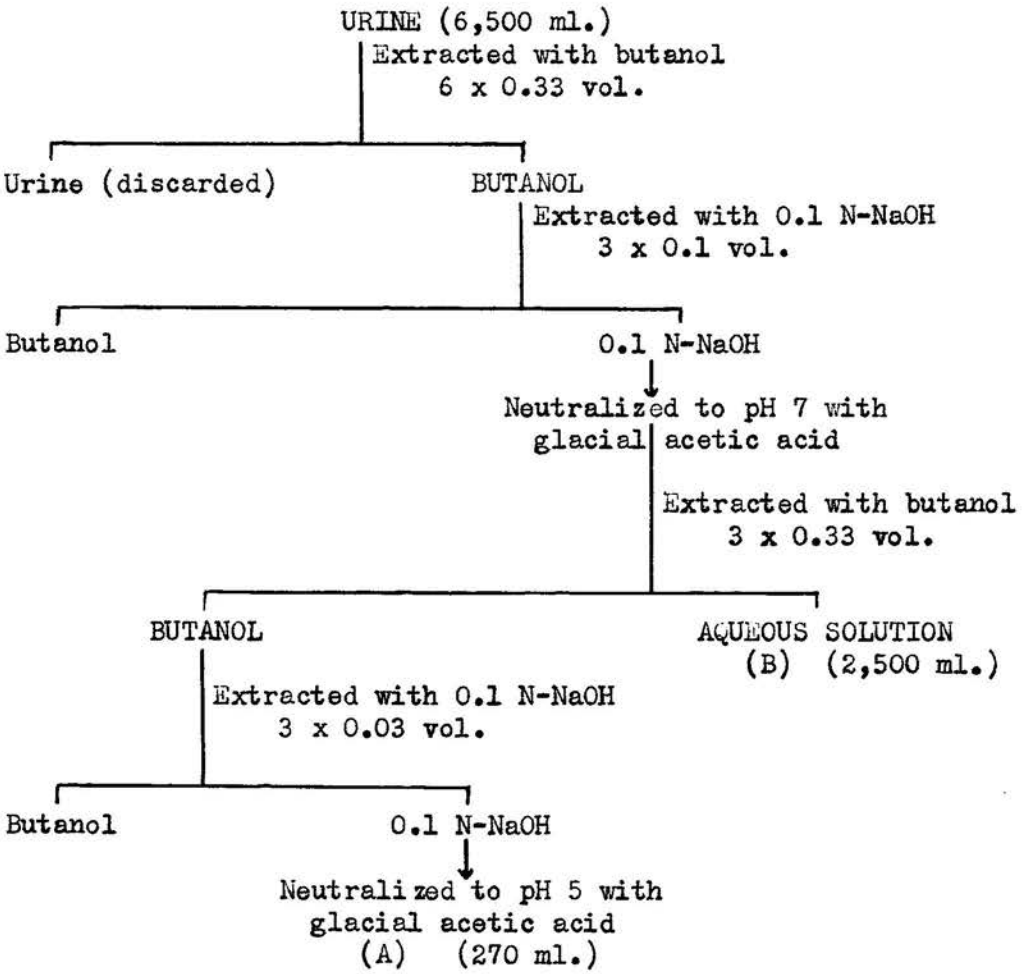
[x] prepared by Dr. E.S.Sutherland.

remarkably high result by comparison with the FS liberated by acid hydrolysis (0.19 mg./l.) suggested the presence of large amounts of corticoid glucuronides in the extracts. A further experiment was carried out to confirm this result.

6,500 ml. pooled urine collected over n-butanol were processed according to the following scheme:



Samples of extracts (A) and (B) were incubated with β-glucuronidase [x] for 26 hr. at 37°. At the same time 'enzyme blanks; /

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[x] prepared by Dr. E.S.Sutherland.

blanks,' 'substrate blanks' and acid hydrolyses were carried out as detailed in Table 17. Extraction after the hydrolyses was performed with chloroform (2 x 2 vol.).

Results.

TABLE 17. Comparison of acid and enzymic hydrolysis of conjugated FS extracted from urine by n-butanol

Extract	Hydrolysis mixture	Spekker reading	Formaldehyde per sample (µg.)	Formaldehydogenic steroid (mg./l. urine)
A. 1.	25 ml. extract & 5 ml. water	0.025	1.1	0.02
		lost	-	-
2.	25 ml. water & 5 ml. enzyme	0.035	1.5	0.03
		0.030	1.3	0.02
3.	25 ml. extract & 5 ml. enzyme	0.362 x	16.0 x	0.29 x
		0.425	18.8	0.34
4.	25 ml. extract acidified to pH 1.0 (25°, 26 hr.)	0.047	2.1	0.04
		0.051	2.3	0.04
B. 1.	250 ml. extract & 20 ml. water	0.032	1.4	0.02
		0.036	1.6	0.03
2.	250 ml. water & 20 ml. enzyme	0.027	1.2	0.02
		0.030	1.3	0.02
3.	250 ml. extract & 20 ml. enzyme	0.327	14.5	0.25
		0.331	14.6	0.25
4.	250 ml. extract acidified to pH 1.0 (25°, 26 hr.)	0.042	1.9	0.03
		0.043	1.9	0.03

x very heavy emulsion during chloroform extraction.

The 'blank' values A1, A2, B1 and B2 show that the enzyme alone or the extract alone account for less than 10% of the formaldehyde /

formaldehyde obtained from the enzymic hydrolysis of the extracts. (A3 and B3). Again the enzymic hydrolysis has released much larger amounts of FS than the acid hydrolysis.

The fact that both extracts (A) and (B) contained the same amounts of enzyme-hydrolyzable and acid-hydrolyzable material indicates the inefficiency of the butanol extraction for the conjugates. The figures obtained therefore probably represent only a small fraction of the total conjugated FS originally in the urine. Direct hydrolysis of the conjugates in urine was therefore investigated.

(b) FS extractable from urine after incubation with crude β-glucuronidase.

A 24 hr. specimen was collected, volume 1,100 ml. (RIC). Hydrolysis was carried out on 200 ml. portions of the urine by acidification to pH 1.0 (25°); other 50 ml. portions were incubated at pH 5.0 and 37° with enzyme. FS in chloroform extracts of the urine samples was estimated at various time intervals after the start of the hydrolyses.

The estimations done on the enzymic hydrolyses were read against an 'enzyme blank' incubated for 96 hr. (37°); the blank contained a negligible amount of formaldehyde.

Results./

Results.

TABLE 18. Comparison of acid and enzymic hydrolyses of conjugated FS in urine

Hydrolysis conditions	Duration of hydrolysis (hr.)	Spekker reading	Formaldehyde per sample (µg.)	Formaldehydogenic steroid (mg./l.)
Untreated	-	0.112	4.95	0.29
pH 1.0, 25°	0.5	0.180	7.96	0.48
pH 1.0, 25°	23	0.241	10.7	0.65
pH 5.0, 37° with enzyme	0.5	0.174	7.69	1.86
	2	0.210	9.28	2.25
	4	0.242	10.7	2.59
	23	0.341	15.1	3.65
	48	0.506	22.4	5.42
	96	0.535	23.7	5.73

Incubation with the β-glucuronidase preparations appears to have liberated amounts of FS about 6 times [x] that obtained after acid hydrolysis and equivalent to an excretion of about 3.7 mg./24 hr. formaldehydogenic steroid. There is little increase in enzyme liberated FS after 48 hr.

The enzymic hydrolysis was repeated on a sample of pooled urine (800 ml.). 550 ml. urine was incubated at pH 5.0 (37°) with β-glucuronidase. Samples equivalent to 50 ml. or 20 ml. urine were withdrawn at various time intervals from the incubation mixture, and assayed for FS. Untreated urine (100 ml.) was assayed as a 'control'. The assays of the enzymic hydrolyzates were read against an 'enzyme blank' incubated 48 hr., 37°.

Results./

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[x] after 23 hr. hydrolysis.

Results.

TABLE 19. Enzymic hydrolysis of urinary conjugates of FS

Time incubated with glucuronidase (hr.)	Sample volume (ml.)	Spekker reading	Formaldehyde per sample (µg.)	Formaldehydogenic steroid (mg./l.)
Untreated urine	100	0.080	3.54	0.39
0.25	50	0.210	9.28	2.04
0.75	50	0.240	10.6	2.33
1.0	50	0.210	9.28	2.04
1.5	50	0.227	10.0	2.20
2.0	50	0.252	11.1	2.44
3.0	50	0.272	12.0	2.64
4.0	50	0.333	14.7	3.23
6.0	50	0.327	14.5	3.19
8.0	50	0.325	14.4	3.17
24	20	0.242	10.7	5.89
48	20	0.247	10.9	6.00

This experiment demonstrates the rapid release of FS by the enzyme action. It seems clear that large amounts of urinary corticoid conjugate can be hydrolyzed by crude enzyme preparations rich in β-glucuronidase. At least some of the CICS is enzyme-hydrolyzable as was shown in the experiments with butanol extracts of urine. It was therefore of interest to investigate the effect of the enzyme preparation on the chloroform soluble conjugates (CSCS).

(c) Effect of β-glucuronidase on CICS and CSCS fractions.

Dr. J.Y.F.Paterson carried out the extraction, hydrolysis and assay of the CSCS whilst the author ran a parallel experiment on the CICS. A 24 hr. urine specimen 1,200 ml. (JYFP) was/

(JYFP) was used. A portion (900 ml.) was acidified to pH 1 and extracted with chloroform (2 x 1,500 ml.). The CSCS was removed from the chloroform by extracting with N-K_2CO_3. Acid and enzymic hydrolyses were carried out on portions of the K_2CO_3 extract. Enzyme and substrate blanks were run simultaneously.

Portions of the urine freed from CSCS and free steroid were taken to pH 5 and incubated with β-glucuronidase; other portions were allowed to stand at pH 1. Samples equivalent to 6.0 ml. and 60 ml. urine were withdrawn from the enzymic and acid hydrolysis mixtures respectively and assayed (Table 20).

TABLE 20. Acid and enzymic hydrolysis of CICS in urine

Sample	Duration of hydrolysis (hr.)	Spekker reading	Formaldehyde per sample (μg.)	Formaldehydogenic steroid (mg./24 hr.)
Enzyme blank	48	0.009	0.40	0.9
Urine blank	48	0.002	0.09	0.2
Enzymic hydrolysis	24	0.056	2.48	5.5
	48	0.075	3.32	7.3
Acid hydrolysis	48	0.039	1.72	0.4
	48	0.041	1.81	0.4

Similar results were obtained when the above experiment (with slight modifications) was repeated on another urine specimen, but in neither case did the CSCS appear to be hydrolyzed by β-glucuronidase. In the above experiment Dr. Paterson found 0.55 mg./24 hr. formaldehydogenic steroid in the CSCS fraction using acid treatment, but enzymic incubation yielded only the equivalent of 0.015 mg./24 hr. formaldehydogenic steroid./

steroid.

These experiments demonstrate a further sharp difference between the CSCS and CICS in urine. The former is not hydrolyzed by β-glucuronidase preparations, whereas the latter yields much larger amounts of free FS on incubation with β-glucuronidase than can be obtained by acid hydrolysis.

Although incubation of urine with β-glucuronidase preparations frees larger amounts of FS than acid hydrolysis, there is no evidence to indicate whether or not these methods of hydrolysis are concerned with the same conjugates. The discrepancy in the amounts of FS obtainable by the two methods of hydrolysis may be due to (a) specificity of the hydrolysis techniques for different conjugates (b) destruction under the conditions of acid hydrolysis of the free FS liberated.

Hence some experiments were carried out to investigate these possibilities.

(d) Acid stability of FS obtained after acid and after enzymic hydrolysis of urinary conjugates.

The following hydrolytic treatments were carried out on portions of a 24 hr. urine specimen 1,100 ml. (RIC).

A. acid hydrolysis.

B. enzymic hydrolysis followed by acid hydrolysis.

C. acid hydrolysis followed by enzymic hydrolysis.

First, free steroid and CSCS were removed by acidification/

acidification of the urine to pH 1.1 and extraction with chloroform (3 x 2 vol.). This 'stock urine' was then processed in the above three ways.

(A) acid hydrolysis: a sample (700 ml.) of the 'stock urine' (pH 1.1) was kept at 25° and portions (100 ml.) withdrawn 2.5, 6, 12, 25, and 48 hr. after acidification. One sample (100 ml.) was withdrawn after 25 hr. hydrolysis and used in (C).

(B) enzymic hydrolysis followed by acid hydrolysis: acidified 'stock urine' (400 ml.) was taken to pH 5.0 immediately after the extraction of the free corticoid and CSCS. The solution was hydrolyzed with β-glucuronidase at 37°. Total volume of the hydrolysis mixture was 610 ml. Samples (10 ml.) were withdrawn 12, 25 and 48 hr. after addition of β-glucuronidase. The remaining 580 ml. was then extracted with chloroform (3 x 0.33 vol.) to remove FS liberated during enzymic hydrolysis. The solution was acidified to pH 1.1 and allowed to stand for 10 hr. at 25°. Samples (100 ml.) were then withdrawn for assay (total volume 600 ml.).

(C) acid hydrolysis followed by enzymic hydrolysis: after 25 hr. acid hydrolysis a 100 ml. sample of (A) was adjusted to pH 5.0 and incubated at 37° with β-glucuronidase. Total volume of the hydrolysis mixture was 140 ml. After 24 hr., 20 ml. samples were withdrawn for assay.

Results/

Results.

TABLE 21. Acid and enzymic hydrolysis of urinary conjugated FS.

Method of hydrolysis of urinary conjugates	Duration of hydrolysis (hr.)	Spekker reading	Formaldehyde per sample (µg.)	Formaldehydogenic steroid (mg./24 hr.)
(A) 1. Acid	2.5	0.075	3.31	0.40
2. Acid	6	0.080	3.54	0.43
3. Acid	12	0.090	3.98	0.48
4. Acid	25	0.090	3.98	0.48
5. Acid	48	0.084	3.71	0.45
(B) 1. Enzymic	12	0.060	2.65	4.89
2. Enzymic	25	0.092	4.07	7.51
3. Enzymic	48	0.145	6.41	11.8
4. Enzymic	48	0.218	9.62	1.84
5. then acid	10	0.222	9.82	1.88
(C) 1. Acid	25	0.058	2.56	2.17
2. then enzymic	24	0.064	2.83	2.40

From these results the following figures can be derived.

Acid hydrolysis, 25 hr.	(A4)	0.48
Enzymic hydrolysis, 25 hr.	(B2)	7.51
Enzymic hydrolysis, 24 hr. (after 25 hr. acid hydrolysis)	(C1 - A4)	1.69
	(C2 - A4)	1.92
Acid hydrolysis, 10 hr. (after 48 hr. enzymic hydrolysis)	(B4)	1.84
	(B5)	1.88

The large amount of FS apparently obtained by acid hydrolysis following enzymic hydrolysis was probably due to incomplete extraction of enzyme-liberated FS. Alternatively, it might have been formed from material in the enzyme preparation during the acid hydrolysis. Hence, from B4 and B5 no conclusion can be drawn as to the existence of conjugates not hydrolyzed by β-glucuronidase /

β-glucuronidase but hydrolyzed by acid.

In this experiment when acid hydrolysis preceeded the enzymic treatment, there was a marked reduction in the amount of FS liberated by the enzyme. This would seem to indicate that the acid conditions (a) altered the 'glucuronide' conjugate(s) so that they were no longer hydrolyzed by the β-glucuronidase, or (b) after hydrolyzing the 'glucuronide' conjugates destroyed the free FS.

The possible destruction in acid solution (pH 1) of the FS liberated by enzymic hydrolysis of urinary CICS was investigated further. A 24 hr. urine specimen, 825 ml.(JYFP) was extracted at pH 2 with chloroform (3 x 0.33 vol.) to remove free FS and CSCS. A sample (400 ml.) was incubated with β-glucuronidase at pH 5.0 for 48 hr. and then extracted with chloroform (3 x 0.5 vol.). The residue after drying and distilling off the chloroform was leached with 60 ml. dry benzene. The residue obtained by evaporation to dryness of 30 ml. of the benzene solution was taken up in almost 400 ml. water. After acidification of the aqueous solution to pH 1.0 the volume was made up to 400 ml. Samples were withdrawn for assay immediately, and after 10, 22, 30, 48, 70 and 94 hr. at pH 1.0, 37°.

Results/

Results.

TABLE 22. Effect of acid on FS liberated from urine by incubation with β-glucuronidase

Time at pH 1.0, 37° (hr.)	Sample volume (ml.)	Spekker reading	Formaldehyde per sample (µg.)	Formaldehydogenic steroid (mg./24 hr.)
0	10	0.052	2.31	4.2
0	10	0.055	2.43	4.4
10	10	0.042	1.86	3.4
10	10	0.041	1.81	3.3
22	10	0.035	1.55	2.8
22	10	0.038	1.68	3.0
30	10	0.020	0.88	1.6
30	10	0.022	0.97	1.8
48	25	0.025	1.10	0.8
48	25	0.030	1.32	1.0
70	25	0.030	1.32	1.0
70	25	0.034	1.50	1.1
94	25	0.027	1.19	0.9
94	25	0.030	1.32	1.0

The amount of FS was found to decrease gradually, and after 24 hr. about 50% of the original material had been destroyed. From 48 hr. to 94 hr. there was little detectable change in the level of FS. In this extract there would seem to have been formaldehydogenic substances, some sensitive to acid and some stable to acid (pH 1.0, 37°).

A further sample of 350 ml. of the urine extracted in the above experiment to remove free FS and CSCS was acidified to pH 1 and allowed to stand at 37° for 48 hr.

Material /

Material liberated in this acid hydrolysis was extracted with chloroform (4 x 200 ml.). The aqueous solution was incubated with β-glucuronidase at 37° for 48 hr. and the mixture was re-extracted with chloroform (4 x 200 ml.). After evaporation of the chloroform extract to dryness the residue was leached with benzene, the benzene solution evaporated to dryness, and the residue taken up in 250 ml. dilute acid pH 1.0 as in the previous experiment. Samples (20 ml.) were withdrawn for assay immediately and after standing 36, 48, and 72 hr. at 37°.

Results.

TABLE 23. Effect of acid on urinary FS liberated by enzymic hydrolysis after acid hydrolysis

Time at pH 1.0 (hr.)	Spekker reading	Formaldehyde per sample (μg.)	Formaldehydogenic steroid (mg./24 hr.)
0	0.072	3.18	1.0
0	0.076	3.36	1.1
36	0.072	3.18	1.0
36	0.079	3.49	1.1
48	0.075	3.32	1.1
48	0.070	3.09	1.0
72	0.081	3.58	1.2
72	lost	-	-

There was no apparent destruction at pH 1.0, 37° of this FS liberated by β-glucuronidase. The similarity between the levels of this 'acid stable' FS (1.1 mg./24 hr.) and that obtained in the previous experiment from the same urine sample /

sample (1.3 mg./24 hr.), suggests these may be identical fractions.

These experiments indicate that acid hydrolyzes at least some of the same conjugated FS as are hydrolyzed by β-glucuronidase, but that some of the free FS formed is destroyed at pH 1. This would account, partly at least, for the much greater amount of FS liberated by enzymic hydrolysis as compared with acid hydrolysis.

Somewhat similar experiments with different urine specimens have confirmed the results of these two experiments.

EXPERIMENTAL

(B) Acetaldehydogenic and Formaldehydogenic Substances.

4,6. Acid hydrolysis of conjugated acetaldehydogenic substances.

Acid hydrolysis of conjugated AS in urine was studied in a similar way to that used for the formaldehydogenic conjugates (4,3).

From a pooled urine sample (600 ml.) 50 ml. was withdrawn for assay without any treatment ('unacidified urine'). The remainder was acidified to pH 1.0 and allowed to stand at 25°. After various time intervals samples equivalent to 50 ml. urine were withdrawn, neutralized to pH 7, extracted with chloroform and assayed for AS. The results (Table 24) indicate the rapid hydrolysis of a conjugate, the free AS being stable at pH 1, 25°. [x] There is no indication of two types of conjugate as was shown for the FS (4,1). Similar curves were obtained in other experiments and also in experiments where the urine was extracted at pH 1.

Acid hydrolysis of the conjugated AS was not extensively investigated since experience with urinary FS had indicated the difficulties of interpreting results obtained in such hydrolyses (cf. Discussion).

Results./

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[x] The author has found that pregnane-3α:17α:20α-triol is stable for at least 53 hr. at pH 1, 25°.

Results.

TABLE 24. Amount of AS extractable by chloroform from urine at pH 1, 25° after various time intervals

Time at pH 1 (hr.)	Spekker reading	Acetaldehyde per sample (μg.)
Unacidified urine	0.069	1.9
1	0.197	5.5
1.9	0.180	5.1
3.3	0.190	5.3
4.3	0.206	5.8
5.3	0.195	5.5
7.0	0.198	5.6
24	0.200	5.6

4,7. Optimum pH for enzymic hydrolysis of conjugated AS and FS in urine.

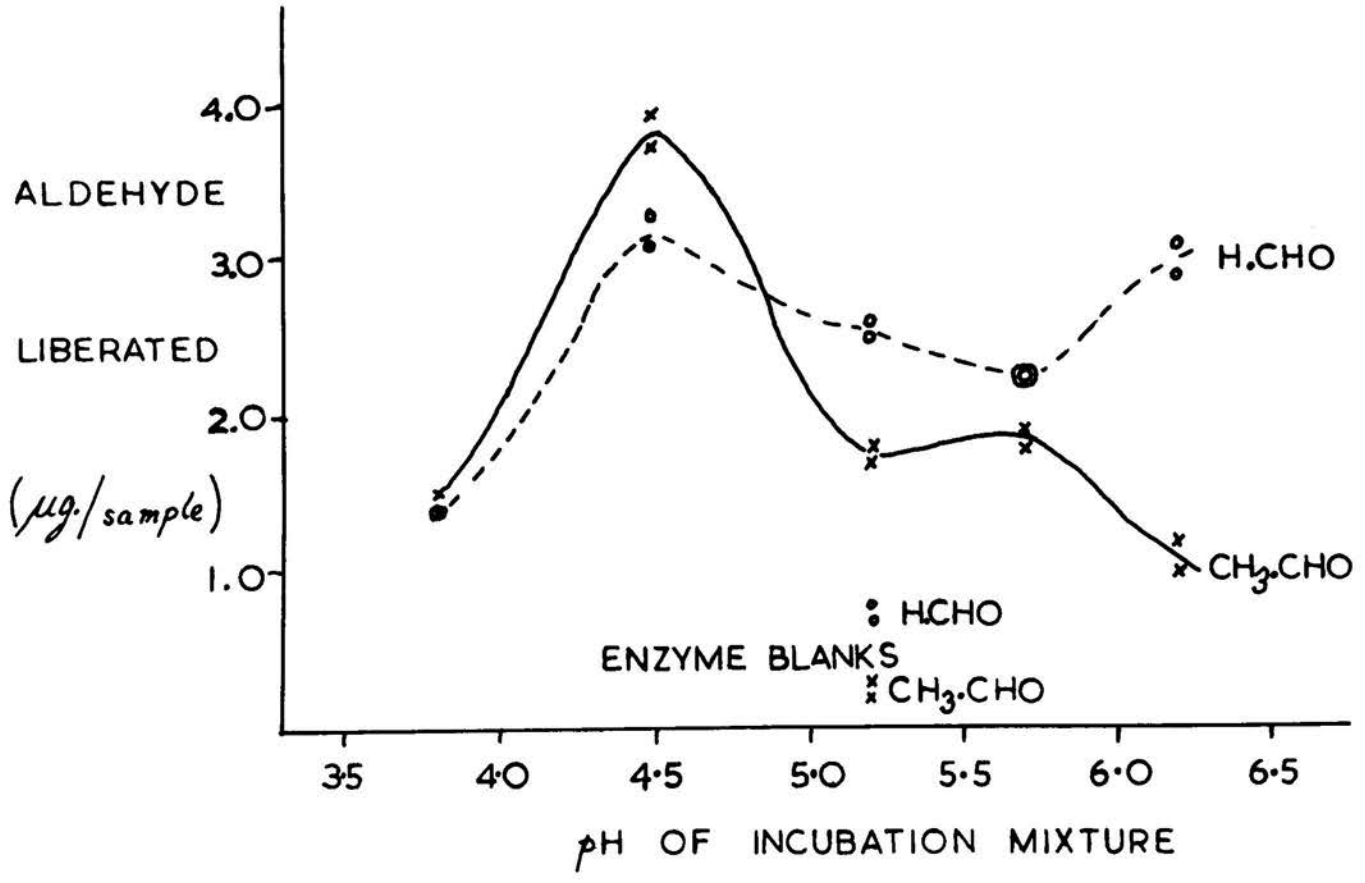
Preceeding experiments demonstrated the effectiveness of enzymic hydrolysis in yielding large amounts of FS. In preliminary experiments using the method described in Section 3 for acetaldehydogenic steroids, similar increases in the amount of AS extractable from urine after enzymic hydrolysis were noted. Experiments were designed to investigate the optimum pH for the enzymic hydrolysis of urinary conjugated AS and FS.

A pooled urine sample was used. Portions of the

urine /

FIG. 9. EFFECT OF pH ON THE ENZYMIC RELEASE OF

ACETALDEHYDOGENIC & FORMALDEHYDOGENIC SUBSTANCES IN URINE



urine (70 ml.) were adjusted to and buffered at various pH values using glacial acetic acid and acetate buffer. After the addition of β-glucuronidase solution the volumes were made up to 120 ml. with water, and the pH of the mixtures noted. The solutions were incubated at 37° for 24 hr. An enzyme blank made up with 5.0 ml. enzyme and 115 ml. 0.1 N-acetate buffer, pH 5.2, was incubated simultaneously. After the incubations the pH values of the solutions were found to be identical to the initial values. Samples of the solutions (30 ml.) were extracted and estimated for AS and FS. (Fig. 9). Little hydrolysis took place at pH 3.8, and there appeared to be a maximum at pH 4.5 for the release of both FS and AS.

In view of the sharp maximum obtained for AS a further experiment was carried out and in this case a series of urine and enzyme blanks also were incubated at various pH values. The mixtures were incubated for only 3 hr. to avoid bacterial contamination and to ensure that hydrolysis was not complete. With this exception the experimental details were essentially as before. The results are given in Table 25. Maximum hydrolysis of conjugated AS again occurred at pH 4.5. Hence in subsequent experiments incubation with β-glucuronidase preparations was carried out at pH 4.5.

TABLE 25./

TABLE 25. Enzymic hydrolysis of urinary acetaldehydogenic conjugates at different pH values

pH of hydrolysis mixtures		Incubation mixture					
		Enzyme + water		Urine + water		Enzyme + urine	
Before incubation	After incubation	Spekker reading	CH_3CHO (µg.)	Spekker reading	CH_3CHO (µg.)	Spekker reading	CH_3CHO (µg.)
4.0	3.96	0.007	0.2	0.063	1.8	0.072	2.0
4.25	4.23	0.006	0.2	-	-	0.070	2.0
4.54	4.51	0.002	0.1	0.059	1.7	0.112	3.1
4.78	4.72	0.000	0.0	-	-	0.081	2.3
5.20	5.16	0.012	0.3	0.059	1.7	0.075	2.1
5.40	5.38	0.010	0.3	-	-	0.055	1.5
5.70	5.68	0.000	0.0	-	-	0.058	1.6

The amounts of acetaldehyde quoted are µg./sample.

4,8. Efficiency /

4,8. Efficiency of enzymic hydrolysis in urine and on n-butanol extracted material.

(a) n-Butanol extraction of glucuronidase-hydrolyzable material from urine; effect of NaOH washing of butanol extracts.

Part of a 24 hr. urine specimen (JYFP) was divided into three 400 ml. portions.

One portion (A) was hydrolyzed directly with β-glucuronidase.[x] Another (B1) was butanol extracted and the extract after evaporation to dryness under reduced pressure[xx] was hydrolyzed in aqueous solution with β-glucuronidase. The third portion (B2) was treated as in (B1) except that the butanol extract was washed with 0.1 N-NaOH.

(A) 400 ml. urine was adjusted to pH 4.5 with acetic acid and the volume made up to 600 ml. Acetate buffer pH 4.5 (60 ml.), enzyme (2,500 G.U., 17 ml.) and 5 ml. chloroform were added and the mixture incubated at 37°. After 24 hr. a further 2,500 G.U. of enzyme were added and the incubation continued for 22 hr. The /

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[x] This urine sample was stored in the refrigerator until B1 and B2 were ready for enzymic hydrolysis.

[xx] Experiments (not reported in this Thesis) with urine extracts and with pure pregnanetriol and 11-deoxycorticosterone showed that urinary PS and AS and the steroids were unchanged by prolonged heating in various solvents at or near neutrality. Thus there did not seem to be any great danger of destruction of corticoid material in evaporating to dryness under reduced pressure neutral butanol extracts of urine.

The mixture was then extracted with chloroform (3 x 600 ml.) and the chloroform extract washed with 0.1 N-NaOH (1 x 0.1 vol.) and water (2 x 0.1 vol.).

(B1) 400 ml. urine, with 33 g. $NaHCO_3$ added, was extracted with n-butanol (3 x 2 vol.). The n-butanol extracts were evaporated to dryness under reduced pressure. Small amounts of n-butanol in the residue were removed by azeotropic distillation with ethanol (3 x 10 ml.) under reduced pressure. The residue was dissolved in 50 ml. water, acidified to pH 4.5 and the volume adjusted to 100 ml. Acetate buffer (10 ml.), enzyme (2,500 G.U.) and 5 ml. chloroform were added. After 24 hr. at 37° a further 2,500 G.U. of enzyme were added and the incubation continued for 22 hr. The mixture was extracted with chloroform (3 x 110 ml.) and the chloroform extracts washed as in (A).

(B2) The procedure was the same as that in (B1) except that the n-butanol extracts were washed with 0.1 N-NaOH (2 x 110 ml.) and water (3 x 110 ml.).

Bad emulsions were encountered in the chloroform extractions of B1 and B2 after incubation with β-glucuronidase, and the results in these cases may therefore be low. Estimations of AS and FS were carried out on portions equivalent to 1/16th of each extract.

Results./

Results.

TABLE 26. Hydrolysis with β-glucuronidase of butanol extracted material from urine

Enzyme incubated with	Acetaldehydogenic substances		Formaldehydogenic substances	
	Spekker reading	Steroid (mg./l.)	Spekker reading	Steroid (mg./l.)
A. Urine	0.151	1.30	0.218	4.24
	0.165	1.42	0.230	4.45
B1. Butanol extract	0.210	1.80	0.228	4.41
	0.229	1.96	0.220	4.28
B2. NaOH-washed butanol extract	0.158	1.36	0.090	1.75
	0.151	1.30	0.090	1.75

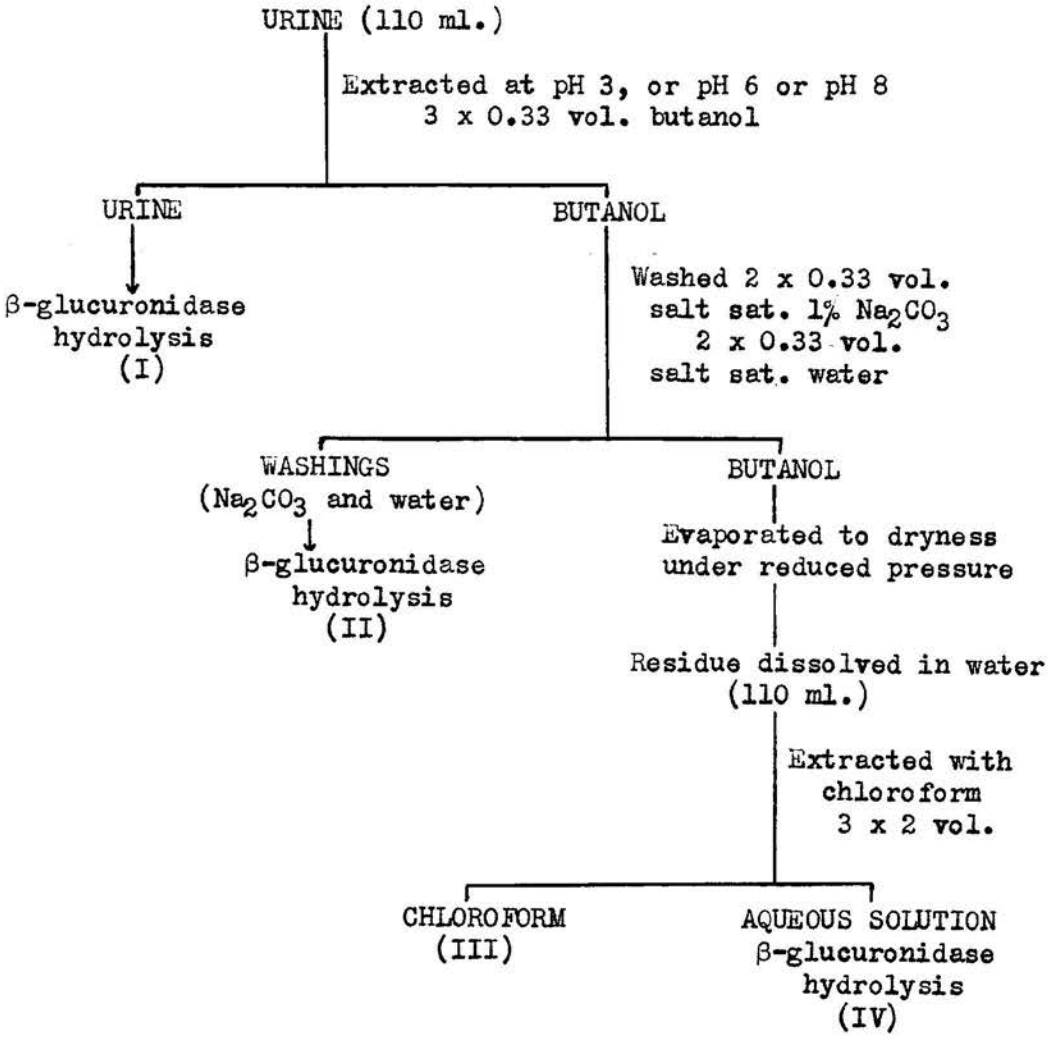
Comparison of A and B1 indicates that enzymic hydrolysis of material extracted from urine by butanol may yield amounts of FS and AS at least as great as direct hydrolysis in the urine. These results might have been due to (a) a higher ratio of substrate concentration to enzyme concentration in B1, or (b) elimination of some enzyme inhibitors by butanol extraction.

NaOH-washing of the butanol extracts was found to result in a loss of 29% of acetaldehydogenic conjugates but 60% of the formaldehydogenic material.

(b) Efficiency of n-butanol extraction of glucuronidase-hydrolyzable material from urine; effect of Na_2CO_3 washing of butanol extracts.

After butanol extraction the amount of enzyme-liberated AS and FS remaining in the urine was estimated. As NaOH washing of the butanol extracts was shown in the previous experiment /

Fractionation of butanol extracts of urine containing conjugated AS and FS



experiment to result in loss of appreciable amounts of conjugates, the use of salt saturated 1% Na_2CO_3 was investigated as a wash stage for butanol extracts.

A 24 hr. urine specimen, 1100 ml. (RIC) was collected over 100 ml. butanol. The butanol extractions of three separate 110 ml. portions of the urine were carried out with the urine samples at the following pH values:

Urine sample	A	B	C
pH of urine before butanol extraction	2.76	6.14	8.40
pH of urine after butanol extraction	3.20	6.32	8.28

The procedure which was carried out for each of the samples A, B and C is shown on the facing page.

The adjustment of the pH of extracts of series I and II to pH 4.5 was carried out immediately after the respective extraction procedures, to minimise destruction or hydrolysis of substances.

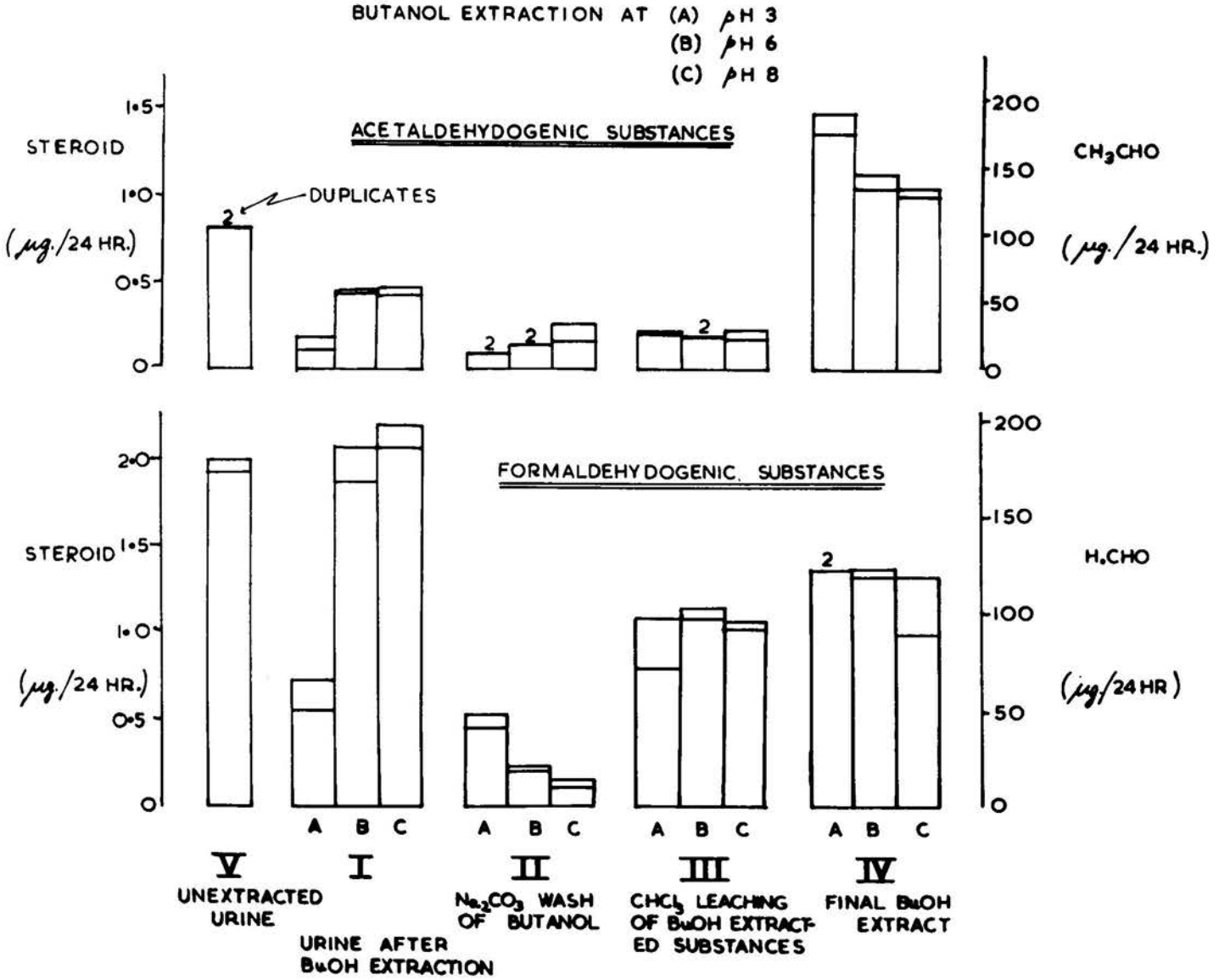
The volumes of the enzyme hydrolysis mixtures were made up to 150 ml. before incubation at 37° for 24 hr. A sample (110 ml.) of untreated urine was hydrolyzed under identical conditions.

Samples equivalent to two-fifths of each hydrolysis mixture and of extract III were assayed for AS and FS (Fig. 10).

The results lead to the following conclusions:

(1) The yield of AS and FS after enzymic hydrolysis of butanol extracts of urine is comparable with that obtained by direct hydrolysis of the urine. /

FIG. 10. DISTRIBUTION OF βGLUCURONIDASE HYDROLYZABLE CONJUGATED AS & FS IN THE FRACTIONATION OF BUTANOL EXTRACTS OF URINE



the urine. There appears to be some dependence on the pH at which the urine is extracted.

(2) Washing the butanol extracts with salt-saturated Na_2CO_3 results in considerable loss of conjugated FS but less AS. (cf. NaOH washing).

(3) Chloroform extracts III A,B,C probably are a measure of free AS and FS in the urine; the levels are independent of the pH at which the urine is extracted as would be expected.

(4) A large amount of material appears to be left in the urine, varying according to the pH at which butanol extraction is carried out. This confirms that such butanol extractions are incomplete. There appears to be a sharp difference between the amounts extracted at pH 3 and pH 6 but little between pH 6 and pH 8.

(5) The sum of material obtained from extracts I, II, III and IV (or even from I and IV) far exceeds that obtained from the unextracted urine. This may be due to the presence of some inhibitor of β-glucuronidase in urine.

4,9. Inhibition of enzyme action with D-glucosaccharic acid.

The enzyme preparations used in these experiments were relatively impure, their high β-glucuronidase activity being only an indication that the urinary AS and FS obtained after incubation with the enzyme preparations were conjugated with glucuronic acid. Karunairatnam & Levvy (1949) have shown that D-glucosaccharic acid is a competitive inhibitor of β-glucuronidase ($Ki = 6 \times 10^{-5}$ M), therefore the effect of saccharic acid on enzymic hydrolysis of urinary conjugates was investigated.

Twelve /

Twelve 24 hr. urine specimens (I - XII) were each treated as follows. Solid $NaHCO_3$ (50 g.) was dissolved in the urine. The mixture was extracted with n-butanol (3 x 1 vol.). The butanol extracts were evaporated to dryness under reduced pressure on a water bath. Butanol was removed from the residue by azeotropic distillation under reduced pressure with ethanol (3 x 10 ml.). The residue was leached with hot dry chloroform (3 x 400 ml.). Chloroform insoluble material was dissolved in 300 ml. water, 30 ml. N-acetate buffer pH 4.5 added and the pH of the mixture adjusted to pH 4.5 with glacial acetic acid. The volume was made up to 450 ml. and the total then divided into three 150 ml. portions which were used to make up incubation mixtures for enzymic hydrolysis.

Incubation mixtures:

A. 150 ml. urine extract, 15 ml. water, enzyme (5,000 G.U.)

B. 150 ml. urine extract, 15 ml. 0.1 M-saccharate [x], enzyme (5,000 G.U.)

C. 150 ml. urine extract, 15 ml. water, boiled enzyme (originally 5,000 G.U.)

D. 155 ml. water, 10 ml. N-acetate buffer, enzyme (5,000 G.U.)

To each of these was added 5 ml. chloroform and they were incubated for 48 hr. at 37°.

After hydrolysis each of the mixtures was extracted with chloroform (3 x 200 ml.). The chloroform solutions were washed /

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[x] Saccharate solutions were adjusted to pH 4.5; final concentration of saccharate in incubation mixture B was 8×10^{-3} M.

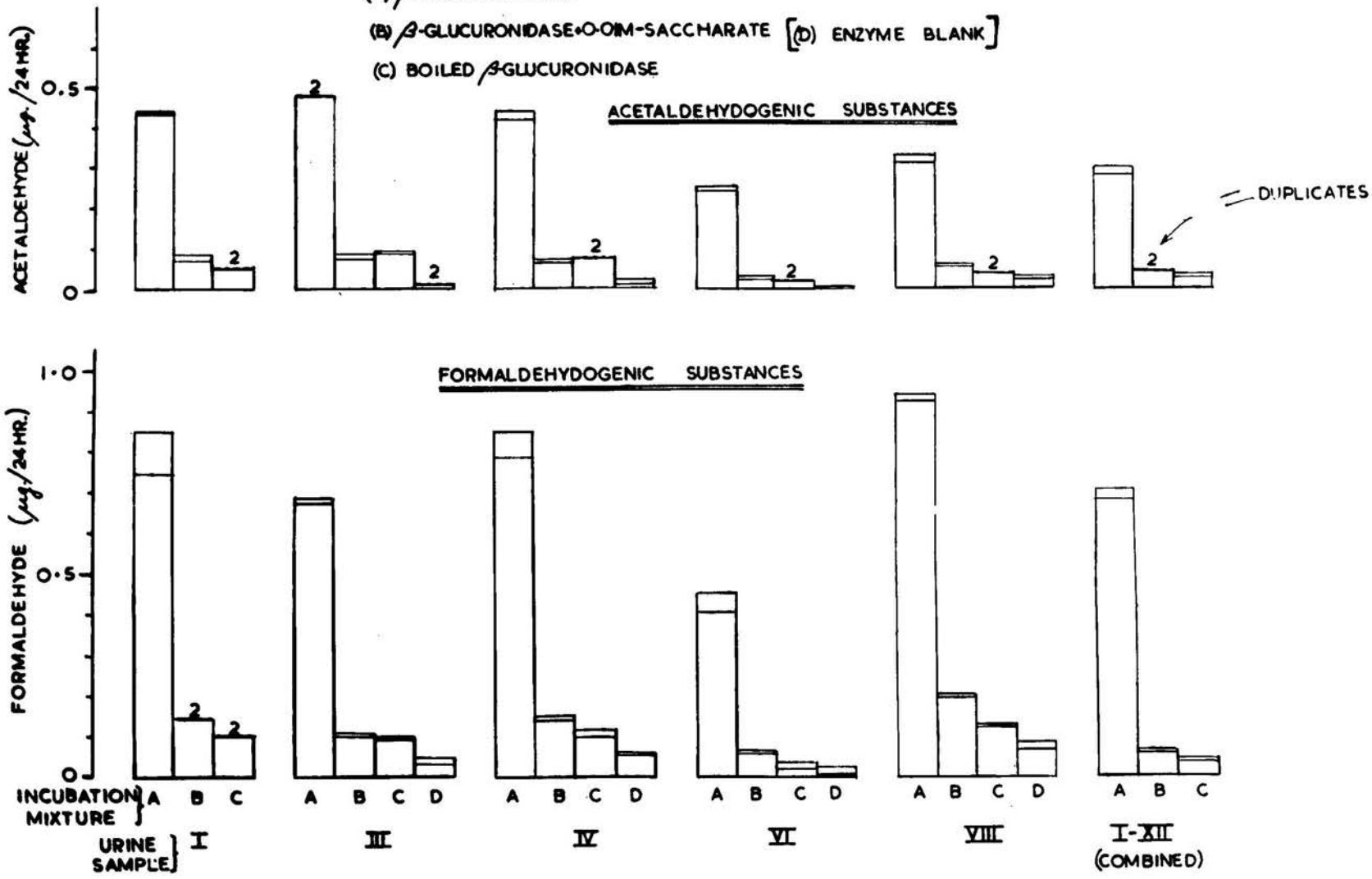
FIG. II

'AS' & 'FS' LIBERATED FROM BUTANOL EXTRACTED MATERIAL FROM URINE BY INCUBATION WITH

(A) β-GLUCURONIDASE

(B) β-GLUCURONIDASE + 0·01M-SACCHARATE [(D) ENZYME BLANK]

(C) BOILED β-GLUCURONIDASE



washed with 0.1 N-NaOH (1 x 200 ml.), the alkali wash being back extracted with chloroform (1 x 100 ml.). This chloroform was added to the main chloroform extract which was then washed with water (3 x 100 ml.). The first two water washes were each back extracted with chloroform (1 x 50 ml.), the latter being added to the main chloroform extract before proceeding to the next wash.

Assays were carried out for AS and FS on extracts from some of the 24 hr. urine specimens, and on the twelve combined extracts of A, B, C and D (Fig. 11). From the results the inhibition by saccharic acid of the enzymic liberation of AS and FS was calculated, as shown in Table 27.

The inhibition appears practically complete, and is strong evidence that the enzyme involved is β-glucuronidase. The latter is specific for β-glucuronides (Masamune, 1934) so it would seem that the conjugates hydrolyzed are β-glucuronides.

TABLE 27. /

TABLE 27. Inhibition of β-glucuronidase liberation of AS and FS with 10^{-2} M-saccharate

Urine batch	Formaldehydogenic substances (mean of duplicate determinations) (mg./24 hr.)				Saccharate inhibition (%)
	A	B	C	D	
III	7.47	1.16	1.08	0.44	99
IV	8.98	1.59	1.19	0.63	95
VI	4.68	0.69	0.30	0.17	91
VIII	10.24	2.17	1.39	0.84	91
I-XII	7.64	0.72	0.52	-	97

Urine batch	Acetaldehydogenic substances (mean of duplicate determinations) (mg./24 hr.)				Saccharate inhibition (%)
	A	B	C	D	
III	3.69	0.63	0.69	0.09	102
IV	3.31	0.54	0.53	0.15	100
VI	1.91	0.22	0.17	0.03	97
VIII	2.50	0.46	0.33	0.24	94
I-XII	2.26	0.37	0.26	-	95

'% saccharate inhibition' is derived from $\frac{A-B}{A-C} \times 100$.

4,10. DISCUSSION.

The two methods of hydrolysis of urinary conjugated FS and AS which were investigated - acid and enzymic hydrolysis - can not be considered quantitative. Acid conditions (pH 1) at 37° were found to be destructive of some free FS in urine or crude urine extracts. However, the stability of AS and FS in acidified urine or urine extracts and the stability of pure steroids may not correspond. Mrs. Michie (unpublished observations) has shown that 11-deoxycorticosterone, 11-dehydro-17-hydroxycorticosterone and 11-deoxy-17-hydroxycorticosterone are stable at pH 1, 25° for 24 hr. and that boiling aqueous solutions at pH 1 for 1 hr. resulted in no destruction of the C-17 side chain of 11-deoxycorticosterone or corticosterone and only 50% destruction of the side chains of the above 17-hydroxysteroids. It is difficult therefore to explain the apparent instability of FS (if steroidal) in urine at pH 1 and 25°, except by assuming that this may be due to presence of other substances.

The acid hydrolyzed AS in urine appeared stable to pH 1, 25°. Pregnane-3α:17α:20α-triol was stable at pH 1, 25° for 53 hr.

Probably not all conjugates are hydrolyzed at pH 1 and 25°; thus sulphates of cholesterol, dehydroisoandrosterone, pregnenolone, chloestan-3α-ol and cholestan-3β-ol are hydrolyzed at pH 1, room temperature [x], whereas pregnane- /

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[x] with simultaneous continuous ether extraction.

pregnane-3α:20α-diol glucuronide is not hydrolyzed under these conditions (Lieberman & Dobriner, 1948). Acid hydrolysis, therefore, may not be applicable to the hydrolysis of all corticoid conjugates.

That it may be of value was shown by Tompsett (1952) (cf. Stewart, Robson & Tompsett, 1952) who used hot acid hydrolysis for the conjugates of C_{21} 17-deoxysteroids which are stable to this treatment, and probably quantitatively hydrolyzed by it. The 17-hydroxysteroids are destroyed by the hot acid conditions.

The effectiveness of β-glucuronidase as a hydrolytic agent was shown by the large amounts of FS and AS which were liberated by the enzyme action.

Large amounts of enzyme-liberated 'reducing corticoids' were first reported by Kinsella, Doisy & Glick (1950) using Escherichia coli preparations. Corcoran, Page & Dustan (1950) obtained similar results with formaldehydogenic substances. Further reports from other workers using bacterial or spleen enzyme preparations have confirmed these observations (Cox & Marrian, 1951; Cohen, 1951a,b; Kinsella, Baggett & Glick, 1951; Daughaday, Farr & Houghton, 1951; Corcoran, Dustan & Page, 1951; Venning, 1952; Bayliss, 1952; Baggett, Glick & Kinsella, 1952).

Proof that these large amounts of enzyme liberated material are indeed steroidal awaits isolation studies. Reports in this field have been published by Lieberman, Hariton & Dobriner (1950), Kinsella, Baggett & Glick (1951), Schneider (1952), Romanoff, Wolf & Pincus (1952) and Baggett, Glick & Kinsella (1952) (see Appendix I).

In /

In developing enzymic hydrolysis as a quantitative hydrolytic technique, the following are amongst the factors which have to be considered:

(1) the concentrations of the steroid conjugate substrates in urine seem to be very small ($M \times 10^{-5}$ at 5.1 mg./l. for a hypothetical deoxycorticosterone glucuronide) and hence enzymic hydrolysis is likely to be of minimal efficiency. For oestriol glucuronide $Km = 5 \times 10^{-4}$ (Fishman, 1939);

(2) other non-steroidal glucuronides are present in relatively large amounts in urine; the total amount of urinary conjugated glucuronic acid appears to be about 1 g./24 hr. Many of these glucuronides may compete for the enzyme;

(3) variable amounts of impurities present in enzyme preparations may affect enzymic hydrolysis;

(4) the amounts of enzyme inhibitors present may vary from urine to urine. Fishman, Altman & Springer (1948) found spleen-glucuronidase activity was frequently inhibited in the presence of urine. In this connection it should be noted that Karunairatnam & Levvy (1949) found that D-glucuronic acid inhibited β-glucuronidase, 100% inhibition occurring at a concentration of 3×10^{-2} M-glucuronic acid. Glucuronic acid released from conjugated β-glucuronides (steroidal or otherwise) by the β-glucuronidase may therefore prevent complete hydrolysis of steroid glucuronides;

(5) as no corticoid conjugates have been isolated yet, the activity of β-glucuronidase preparations must necessarily be estimated /

estimated with respect to other substrates such as phenylglucuronide or phenolphthalein glucuronide.

Therefore, no attempt was made by the author in these preliminary experiments to obtain maximum enzymic hydrolysis by increasing the enzyme concentration as it was felt that such a maximum would not represent quantitative hydrolysis. There is no evidence to indicate that any of the procedures used for enzymic hydrolysis gives more than comparative values. Venning (1952) studied the hydrolysis of urinary conjugates, measuring liberated biologically active or formaldehydogenic substances and substances giving the Porter-Silber reaction (1950). After using acid hydrolysis and hydrolysis with ox-spleen or Escherichia coli preparations of β-glucuronidase, Venning concluded that there was no satisfactory method for the quantitative hydrolysis of urinary conjugated corticoids at present.

When using preparations of β-glucuronidase for enzymic hydrolysis it must be remembered that there may be other conjugates not hydrolyzed by the enzyme preparations or that the preparations may be active in the hydrolysis of conjugates other than β-glucuronides. Buehler et al. (1949, 1951) and Kinsella et al. (1950, 1951) have shown that their bacterial glucuronidase preparations do not contain phenol- or alcohol-sulphatases. In the author's experiments, saccharic acid (8×10^{-3} M) gave 91 - 102% inhibition of the enzymic hydrolysis /

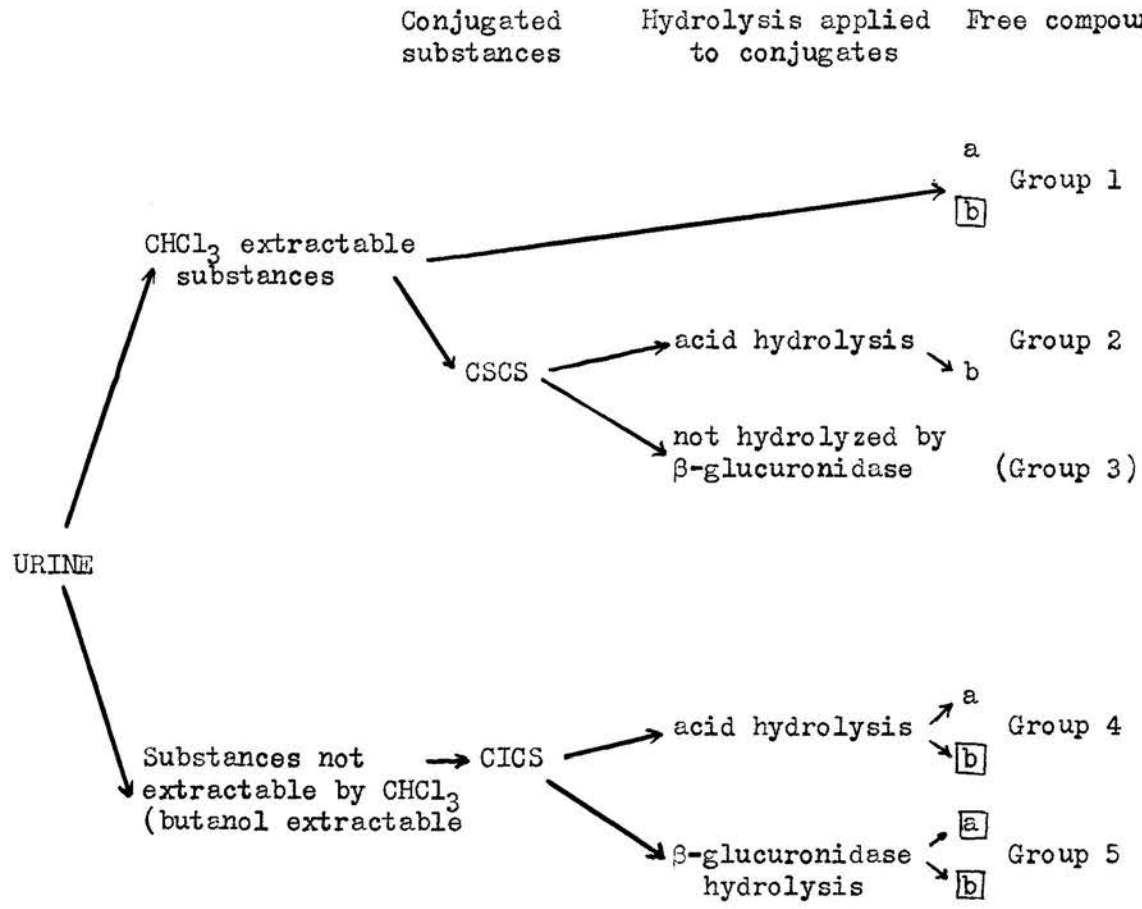
hydrolysis of conjugated AS and FS, and it is therefore unlikely that enzymes other than β-glucuronidase were involved. Dr. A.B. Roy found negligible sulphatase activity in a standard β-glucuronidase preparation used by the author. Thus the conjugates hydrolyzed are probably β-glucuronides as the enzyme β-glucuronidase is specific for such conjugates (Masamune, 1934). This very specificity implies that conjugates other than glucuronides would not be hydrolyzed by such enzyme preparations.

It should be noted that bacterial β-glucuronidase from Escherichia coli and the ox-spleen β-glucuronidase do not appear to be the same; they have pH optima of 6.2 and 4.5 & 5.2 respectively in the hydrolysis of phenolphthalein glucuronide. Also their behaviour towards protein precipitants differs (Doyle & Katzman, 1952).

An optimum pH of 4.5 was found for the enzyme release of AS and FS from urine. Cohen (1951b) quotes pH 5.2 as the optimum for formaldehydogenic substances (using calf-spleen enzyme preparations). Bayliss (1952) with rabbit liver or spleen β-glucuronidase used pH 4.5 for hydrolyses.

The behaviour of conjugated FS and AS to n-butanol extraction of urine paralleled that shown by oestriol glucuronide: they were extracted more readily from acid solution, and re-extracted from n-butanol by NaOH solutions. n-Butanol was found to be less efficient for extracting the conjugated FS and AS than it is for oestriol glucuronide. This may be due in part to the more polar nature of the steroid moeity of corticoid conjugates. Grant & Beall (1950) showed that cyclohexanol /

FIG. 12. Formaldehydogenic substances extractable from the urine of normal human subjects



(a) compounds which may be extracted with difficulty by chloroform from aqueous solution;

(b) compounds readily extracted by chloroform from aqueous solution;

☐ these groups include adrenocortical steroids isolated from urine (Appendix I).

Notes: some conjugates may be common to Groups 4 and 5;

acetaldehydogenic substances of Groups 1a,b, 4, 5a,b appear to be present.

cyclohexanol was more efficient than n-butanol for the extraction of oestrogen conjugates. Investigation of solvents other than n-butanol may prove of value for the extraction of corticoid conjugates.

It must be emphasised that great caution is necessary in the interpretation of results obtained in the hydrolysis of urinary conjugated FS and AS. From the work carried out by the author and by Dr. J.Y.F.Paterson in this laboratory AS and FS in urine may be classified as in Fig. 12. Fractions from which C_{21} adrenocortical steroids have been isolated (Appendix I) are boxed in.

Dr. Paterson found that acid hydrolysis of CSCS was very rapid in urine at pH 1, 25° (about 2 hr.) but appeared less rapid as the conjugate fraction was purified; some purified fractions showed little hydrolysis in 2 hr. at pH 1, 25°. Thus, the apparent rates of hydrolysis of the urinary conjugates by acid may be markedly dependent on the presence of other substances (cf. instability of FS in crude urine extracts, Section 4,5d)

The extraction of free corticoids from urine by chloroform (before or after hydrolysis) may be satisfactory for steroids as polar as 17-hydroxy-11-dehydrocorticosterone (Table 2, Section 1,2) but no data have been published as to the efficiency of extraction procedures for more polar compounds. FS and AS slowly extracted from neutral urine by continuous chloroform extraction have been found by Dr. Paterson and the author /

author (Group Ia, Fig. 12). Even after 24 hours continuous extraction these compounds are incompletely removed. Such substances may not be steroidal and recent work by Dr. Paterson strongly suggests that non-steroidal α-glycols account for most of these FS and AS.

However, the formaldehydogenic and reducing substances liberated by enzymic hydrolysis are mainly more polar substances than 17-hydroxy-11-dehydrocorticosterone. (Group 5a, Fig. 12) Thus, Corcoran, Dustan & Page (1950) found compounds of the $C_{21}O_5$ group, and Baggett, Glick & Kinsella (1952; see also Kinsella, Baggett & Glick, 1951) obtained mainly pregnane-3α:17α:21-triol-11:20-dione after glucuronidase hydrolysis of conjugates in the urine of normal adults and pregnant women. Romanoff, Wolf & Pincus (1952) obtained allopregnane-3β:11β:17α:21-tetrol-20-one after β-glucuronidase hydrolysis. The author has obtained evidence from partition chromatograms as to the polar nature of the major portion of β-glucuronidase-liberated FS from urine (Section 5,4). Robinson & Norton (1951) noted that neutral reducing substances were incompletely removed from urine (after hydrolysis at pH 1, room temperature, for 1 - 11 days) by repeated chloroform extraction.

Some of the more slowly chloroform extractable fractions, if they contain corticoids, require more efficient methods /

methods of extraction for quantitative studies; on the other hand,if non-steroidal,the removal of such substances from urine extracts is necessary in order to avoid interference in the estimation of the steroidal fractions. These difficulties make it imperative that proof of the nature of substances in the various fractions be obtained before the hydrolysis of urinary corticoid conjugates can be perfected.

A field which deserves more attention is the extraction or separation from urine and partial purification of corticoid conjugates themselves before hydrolysis. It may even be possible to estimate the conjugates directly and avoid the difficulties of quantitative hydrolysis. Jayle and co-workers (review: Jayle & Crepy, 1952) have carried out research on the clinical significance of steroid conjugate fractions extractable from urine at different pH values by n-butanol.

SUMMARY. /

SUMMARY.

1. In urine several groups of conjugated acetaldehydogenic and formaldehydogenic substances were found, some of which may be hydrolyzed at pH 1, room temperature, and some by β-glucuronidase preparations.

2. Hydrolysis with crude ox-spleen β-glucuronidase yields much larger amounts of AS and FS than acid hydrolysis; acid conditions may be destructive of some of the FS of urine.

3. The optimum yield of free AS and FS was obtained by β-glucuronidase hydrolysis of urinary conjugates at pH 4.5 (37°).

4. The release of AS and FS from urine by crude β-glucuronidase is almost completely inhibited by 8×10^{-3} M-saccharic acid indicating that these effects are indeed due to hydrolysis of β-glucuronides.

5. It was found possible to extract chloroform insoluble conjugated AS and FS from urine at pH 3 - 8 with n-butanol.

6. Hydrolysis with β-glucuronidase of conjugated AS and FS appears to be more efficient when applied to butanol extracted material.

SECTION 5.

PARTITION CHROMATOGRAPHY OF PURE STEROIDS AND OF URINE EXTRACTS ON HYFLO SUPERCEL COLUMNS.

5, 1. INTRODUCTION.

Adsorption chromatography of C_{21} adrenocortical steroids has not been widely applied until recently. Alumina, used so successfully by Lieberman & Dobriner (1945) and Dobriner, Lieberman & Rhoads (1948) for ketosteroids, appears destructive of some free adrenocortical steroids (Genest, 1951). Adsorption of the steroid acetates on magnesium silicate-celite mixtures has been used in preparative studies (Schneider, 1950b,c, 1951, 1952). Adsorption chromatography of the free steroids has been carried out by Pincus & Romanoff (1950) and Romanoff et al. (1952) on silica gel columns and by Nelson & Samuels(1952) on florosil and magnesium silicate - celite columns. Some disadvantages of adsorption chromatography have been noted by Bush (1952).

Paper partition chromatography of adrenocortical steroids was developed by Zaffaroni et al. (1950), using the solvent systems formamide/benzene and 1:2-dihydroxypropane/toluene. Butt et al. (1951) found that aqueous methanol/n-hexane systems on Hyflo Supercel columns were suitable for progesterone, androst-4-ene-3:17-dione, testosterone and 11-deoxycorticosterone.

It was decided to investigate partition chromatography on columns rather than on paper as it was expected that the former would lend themselves more readily to quantitative studies and could be adapted for large scale isolation work on urinary corticoids. The 1:2-dihydroxypropane used in the system /

system of Zaffaroni et al. (1950) would be expected to give, on oxidation with periodic acid, formaldehyde and acetaldehyde; formamide may be readily hydrolyzed by moisture. The fact that formamide, toluene and 1:2-dihydroxypropane have boiling points higher than 100° makes their removal from column eluates difficult. Thus, for chromatography on Supercel columns the development of a solvent system and technique similar to those of Butt et al. (1951) was indicated. More polar solvents than n-hexane were necessary for the C_{21} adrenocortical steroids and 50 - 90% aqueous methanol/benzene, benzene-hexane or benzene-chloroform systems have been investigated by the author. Pregnane-3α:17α:20α-triol was the main steroid used in developing suitable solvent systems because it was desired to investigate the possible presence of this steroid in urine; also, its polarity is such that an effective partition system for pregnanetriol is likely to be applicable to many adrenocortical steroids. The system 80% aqueous methanol/benzene has been found suitable on Supercel columns for pregnane-3α:17α:20α-triol, 11-deoxycorticosterone, 17-hydroxy-11-dehydrocorticosterone and 17-hydroxy-11-deoxycorticosterone. Purification of urine extracts and separation of at least three groups of acetaldehydogenic compounds present has been possible with the partition chromatograms. No thorough investigation into quantitative recovery of steroids from the partition columns has been made, but results show that good recoveries are possible.

5, 2. MATERIALS /

5, 2. MATERIALS AND METHODS USED.

The methods used for purifying the supporting phase and preparing the column were adapted from those used by Butt et al. (1951).

(a) Purification of Hyflo Supercel. Supercel was covered with concentrated HCl, A.R., the mixture was stirred up thoroughly and left to stand 12 hr. The yellow HCl solution was sucked off on a sintered glass filter and the Supercel washed exhaustively, first with tap-water and then with distilled water, until the filtrate was neutral in reaction (glass electrode). The Supercel was dried in an oven at 110° for about 12 hr., and then baked over a Mekker burner for several hours, with occasional stirring of the powder. The dry purified Supercel was cooled in a vacuum dessicator, and stored in a tightly stoppered bottle until required.

(b) Preparation of columns. Columns were packed in glass tubes of two sizes. For up to about 250 µg. amounts of steroids, tubes were 1 cm. in diameter and 18 cm. long, one end being fitted with a B14 standard joint and the other being flat glass pierced with small holes regularly placed. For larger scale work the glass tubes were 2 cm. in diameter and 50 cm. long. The following description of the packing of a column refers to a smaller column; with a larger column corresponding increases in dimensions and amounts of material apply. The equilibration of solvents,/

solvents, and the preparation and running of columns were carried out in a thermostatically controlled room within the range 15 - 20° and usually at 17.5 ± 0.5°.

Stationary phase (6.0 ml.) was added slowly with thorough mixing to Supercel (6.0 g.). The mixture was stirred to ensure even distribution of the stationary phase and to break up any small lumps. The final mixture still appeared dry. Mobile phase was then added and some of the slurry transferred to the column which was partly immersed in a test tube containing mobile phase. A packer (Howard & Martin, 1950), consisting of a perforated brass disc of slightly smaller diameter than the inside of the column and attached to a long central stainless steel rod, was used to prepare the column. The packer was moved rapidly up and down the column to distribute the slurry as a uniform suspension and to free air bubbles. It was then moved down slowly to trap some of the suspended Supercel and compress it firmly into a pad. The operations were repeated and the column built up slowly, each pad being about 1 mm. thick. The first pad that was formed effectively sealed the holes in the end of the glass tube, and if it had been formed evenly and firmly, no Supercel broke away during the running of the column. The Supercel slurry was gradually packed until the requisite column length was reached.[x] Mobile phase was supplied at constant pressure from a separating funnel /

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[x] Mobile phase, about 10 ml., was allowed to flow through the column to wash it and to check its rate of flow.

funnel fitted with a B14 standard joint and having a capillary inlet.

The columns were packed to a length of 10.0 cm. (in initial experiments 6.3 cm.) and the firmness of packing was such that a flow rate of 7 - 10 ml. per hour was obtained with a head of about 5 cm. mobile phase. Some slight adjustment of the flow rate was possible by varying the head of mobile phase above the column, but if the flow rate was not suitable a fresh column was packed; use of positive or negative pressure to adjust the flow rate was not satisfactory.

(c) Transference of samples to a column. Three methods have been investigated: the sample was

(1) dissolved in mobile phase and run on to the column;

(2) dissolved in stationary phase and added to a small pad of dry Supercel placed on the top of the column (Butt et al., 1951)

(3) dissolved in a suitable organic solvent, dried on to a small amount of Supercel, and the mixture added to the top of the column.

The simplest method is (1) and it has proved generally applicable to urine extracts. The sample was dissolved in 0.5 - 1.0 ml. mobile phase and for quantitative work an accurately measured portion was pipetted on to the column. This was allowed to flow into the column under 1 - 2 cm. Hg positive pressure, and fresh mobile phase (1.0 ml.) added to the top of the column. The liquid was again forced down under slight positive pressure and the process repeated with 1.0 ml. mobile /

mobile phase [x]. After covering the Supercel surface with fresh mobile phase the column was run with mobile phase supplied from the reservoir.

With pure steroids it was sometimes difficult to dissolve the required amount of compound in 0.5 - 1.0 ml. mobile phase. Method (2) was then used. The liquid level above the column was run down, under slight positive pressure, until it was just below the surface of the Supercel and a layer of dry Supercel (0.15 g.) was placed on the top of the column. A portion (0.15 ml.) of a solution of the compound in stationary phase was added to the dry Supercel. Mobile phase was added carefully and the column run as before.

Method (3) did not seem to be quantitative and it was difficult to remove all of the added solvent from the Supercel without excessive heating. It may be of value with bulky extracts for preparative work.

(d) Characteristics of partition columns. Martin & Synge (1941) developed the theoretical basis of partition chromatography by considering partitioning on a column analogous to fractional distillation; the solutes are partitioned between 'plates' of mobile and stationary phase down the length of the column. In deriving /

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[x] During this process the eluate is collected as it is required in the calculation of retention volumes of compounds (see next pages).

deriving equations for the behaviour of the solutes on the chromatograms the following assumptions were made:

(a) diffusion of solute from one 'plate' to another is negligible;

(b) at equilibrium, the distribution ratio of one solute between the two liquid phases is independent, both of the absolute value of its concentration, and of the presence of other solutes.

Then,

$$K = \frac{A}{R.As} - \frac{A_L}{As} \qquad (1)$$

where K = partition coefficient at equilibrium between the two phases,

$$= \frac{\text{concentration of solute in stationary phase}}{\text{concentration of solute in mobile phase}} ;$$

A_S = cross-sectional area of the stationary phase;

A = cross-sectional area of the column;

A_L = cross-sectional area of the mobile phase;

$$R = \frac{\text{movement of peak concentration of the solute band}}{\text{movement of liquid surface above the column}} .$$

Practical limitations noted by Martin & Synge were:

(a) the partition coefficient is seldom constant but decreases as the concentration of solute increases. Hence the front of the band becomes steeper, the back flatter, and the band wider;

(b) interaction between solutes often leads to a greater separation over the theoretical, the more strongly absorbed solute eluting the less strongly absorbed;

(c) non uniformity of flow.

The /

The first limitation is a useful criterion in practice for judging the 'capacity' of a column with a given solvent system and solute, for non-symmetrical elution denotes that the concentration of the solute is too great for ideal chromatography.

Butt et al. (1951) modified equation (1) to:

$$K = \frac{V_R}{L.As} - \frac{A_L}{As} \qquad (2)$$

for $$R = \frac{A.L}{V_R} \qquad (3)$$

where V_R = 'retention' volume or volume of eluate to the peak concentration of the solute;

L = length of the column.

A comparison can thus be made between the partition coefficient of a solute measured directly by equilibration between the phases of the solvent system used and that calculated from its retention volume on a column.

'As' was determined from the dimensions of the columns and the volume of stationary phase/g. Supercel (cf. Martin & Synge, 1941). A_L was obtained as described by Butt et al.(1951).

For chromatograms described in the experimental section the above equations have been used to compare retention volumes of a given substance on different columns. Since R is a constant /

a constant for a given substance and solvent system,

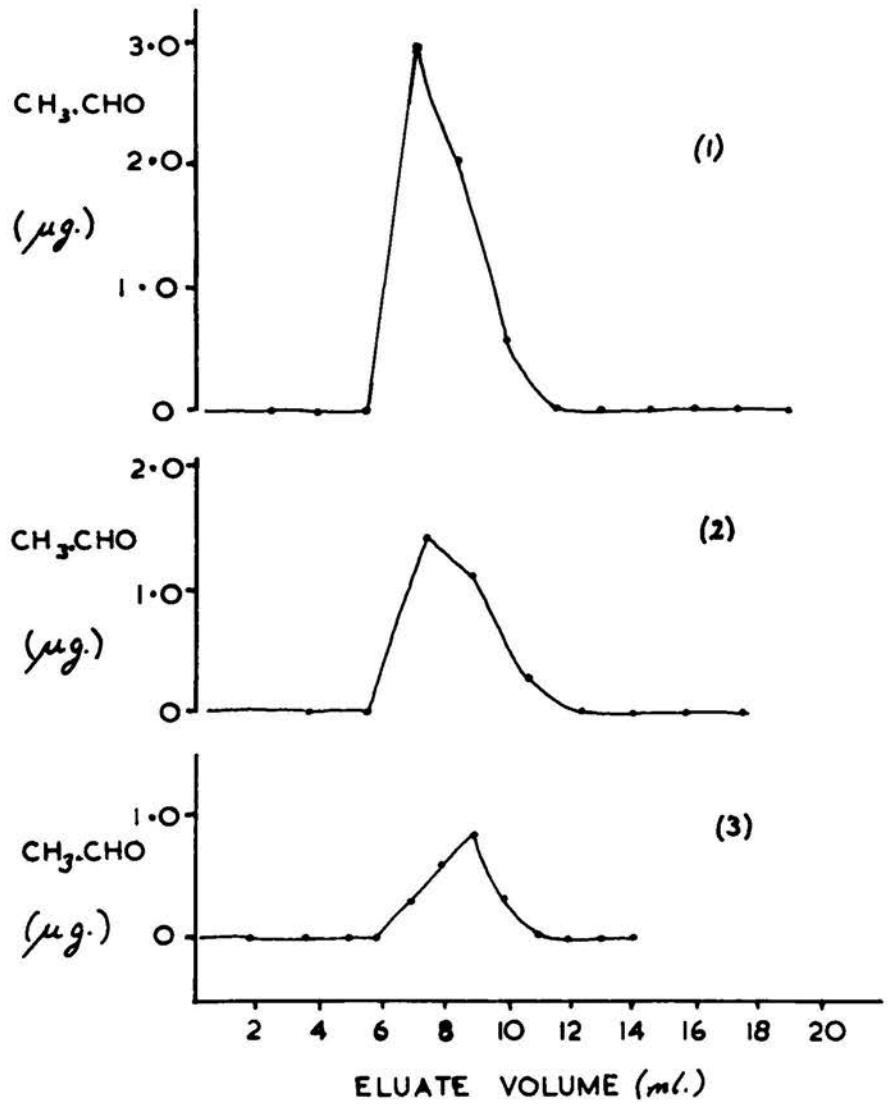
$$\frac{V_R'}{V_R''} \qquad \frac{V_D'}{V_D''} \tag{4}$$

where V_R' , V_R'' are the retention volumes of the substance on columns (') and (") and V_D' & V_D'' are the dead volumes of the columns (the dead volume of a column is equal to the retention volume of a solute of zero partition coefficient). Determination of dead volumes were carried out on some columns after the chromatography of steroids; the dye Sudan III was used as the solute. It was found in chromatographing urine extracts that the front of the first pigment band had a partition coefficient very close to zero with the solvent systems investigated. It was therefore possible to identify the dead volumes by noting the eluate volume at which the urinary pigment band appeared.

Retention volumes of pregnanetriol from column to column with a given solvent system have been consistent where the columns had the same dimensions. Use of equation (4) to relate the known retention volume of a solute on one column to its retention volume on a column of different dimensions gave good agreement with observed values.

5, 3. EXPERIMENTAL./

FIG. 13. PARTITION CHROMATOGRAMS OF PREGNANETRIOL (1 & 2) AND A URINE EXTRACT (ENZYMIC HYDROLYSIS)(3).
SOLVENT SYSTEM: 50% METHANOL / 67% BENZENE – 33% HEXANE



EXPERIMENTAL.

5, 3. Partition chromatography of pure steroids.

Partition chromatogram of pregnanetriol and a urine extract; solvent system aqueous 50% methanol/67% benzene-33% hexane. [x]

In preliminary investigations the following solvents were equilibrated:

15 ml. methanol,
15 ml. water,
100 ml. benzene,
50 ml. hexane.

Pregnanetriol was run on a column with this solvent system, and as the result was promising the run was repeated on fresh columns with pregnanetriol and with an extract prepared from urine hydrolyzed with β-glucuronidase. The columns were 6.3 cm. long. Results are shown in Fig. 13.

The chromatograms indicate the presence of a pregnanetriol-like fraction in the urine extracts. An oily pigment band appeared in the first few eluate fractions whereas the subsequent fractions contained little or no oil or pigment. However, the separation between the pregnanetriol-like fraction and the main pigment band was small, and with such rapid elution the system was found to be of little value for less polar compounds than pregnanetriol.

Therefore, the partition coefficients of pregnanetriol /

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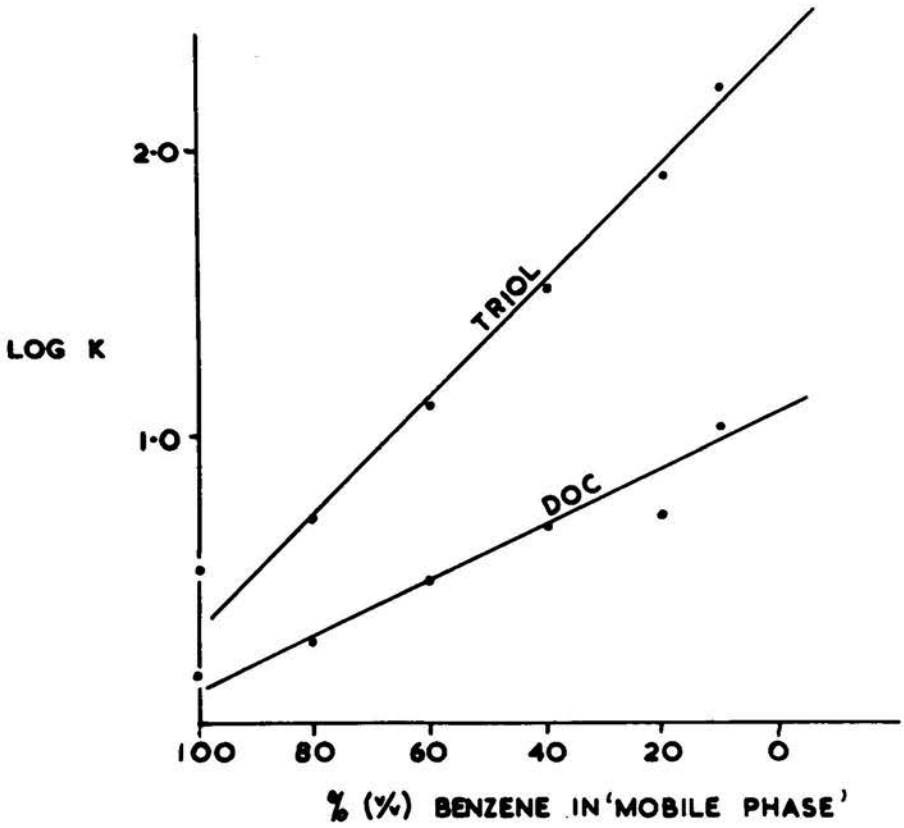
[x] The composition of solvent systems is stated as the proportion (v/v) of the components in each phase before equilibration.

FIG. 14. PARTITION COEFFICIENTS OF PREGNANETRIOL AND

11-DEOXYCORTICOSTERONE, SOLVENT SYSTEM: 70% METHANOL/BENZENE-HEXANE



pregnanetriol and 11-deoxycorticosterone between the phases of different solvent systems were investigated. Known amounts of pregnanetriol and 11-deoxycorticosterone were dissolved in 3.0 ml. of aqueous ethanol or methanol ('stationary phase') and a total of 10.0 ml. of the other solvents added. The mixtures were shaken thoroughly and allowed to equilibrate for at least 4 hr. in a thermostatically controlled room (16 ± 0.5°), then samples of 1.0 ml. and 7.0 ml. were withdrawn from the stationary phase and mobile phase [x] respectively, and assayed. Partition coefficients were calculated from the ratios of the concentrations of acetaldehydogenic and formaldehydogenic steroids in the phases.

Results.

TABLE 28. Partition coefficients of pregnane-3α:17α:20α-triol (Triol) and 11-deoxycorticosterone (DOC) in various solvent systems, at 16 ± 0.5°.

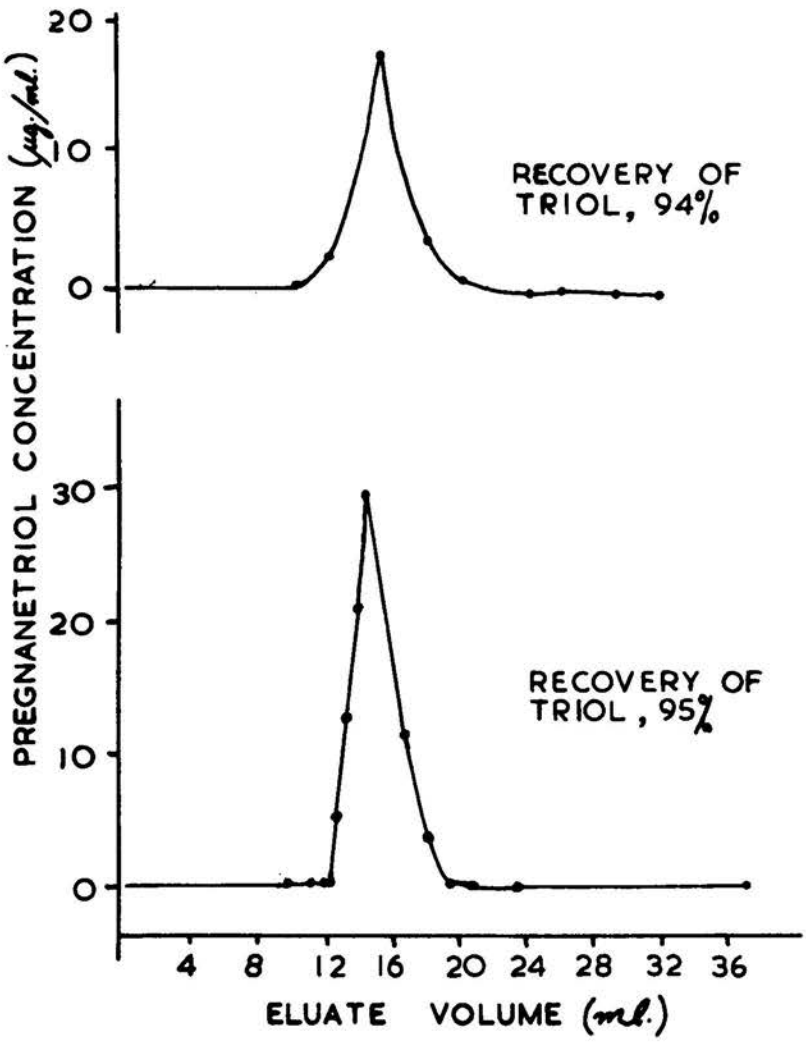
Solvent system	Partition coefficients Triol	DOC
70% ethanol/benzene	1.4	-
70% methanol/cyclohexane	41	-
70% methanol/benzene	3.7, 3.5	1.5
70% methanol/80% benzene-20% hexane	5.3	1.9
70% methanol/60% benzene-40% hexane	13	3.1
70% methanol/40% benzene-60% hexane	32	4.7
70% methanol/20% benzene-80% hexane	80	5.3
70% methanol/10% benzene-90% hexane	170	12.0

In the 70% methanol/benzene-hexane system it appears that /

[x] Aqueous phases correspond to stationary phases on the partition chromatograms used.

FIG. 15. PARTITION CHROMATOGRAMS OF PREGNANETRIOL (75μg).

SOLVENT SYSTEM : 70% METHANOL / BENZENE



that 'mobile solvent' composition $\propto$ log. K as shown in Fig. 14 (cf. Engel, Slaunwhite, Carter & Olmsted, 1951).

On partition columns, systems containing hexane were found to flatten the elution curve of pregnanetriol markedly (5,1d) and so were not used.

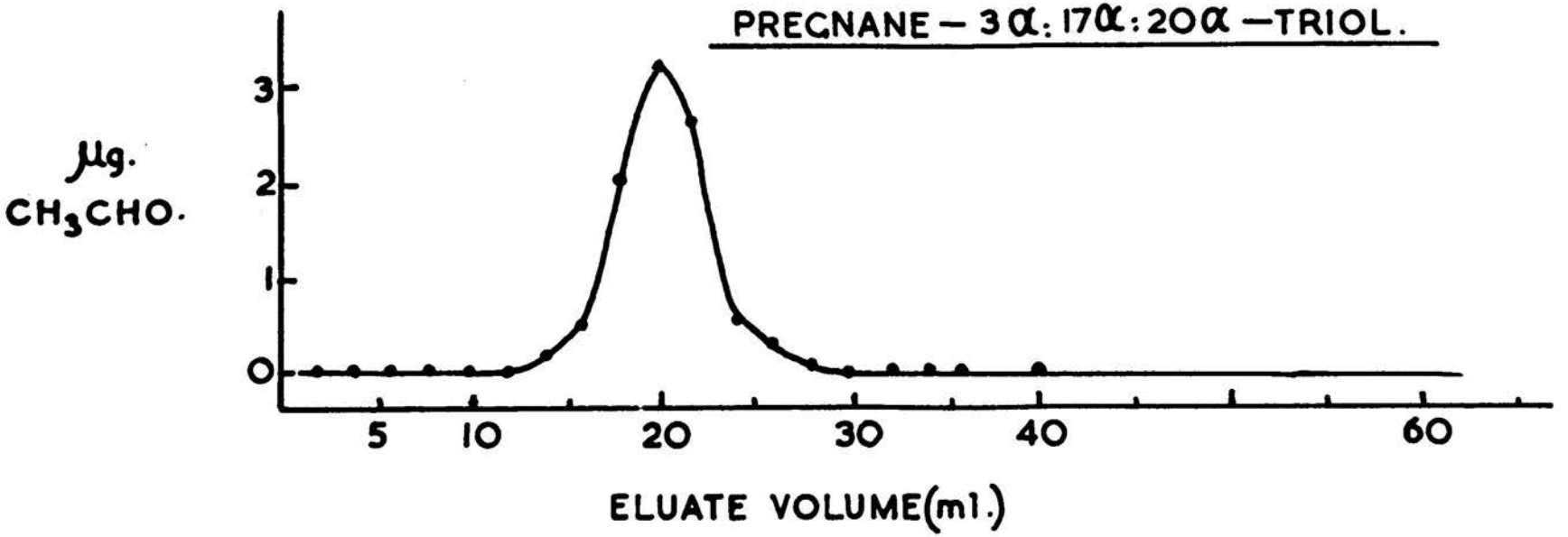
Partition chromatograms of pregnane-3α:17α:20α-triol; solvent system aqueous 70% methanol/benzene.

The retention volume of pregnanetriol was determined in two experiments; in the first, 2.0 - 4.0 ml. fractions were collected to find the approximate retention volume, and in the second, 0.5 ml. fractions were collected where the pregnanetriol was expected (Fig. 15). The recoveries of the pregnanetriol (75 μg.) put into the chromatograms were checked. The recoveries of pregnanetriol indicate that the technique is satisfactory for quantitative work with the pure steroid.

$L = 10.0$ cm., $As = 0.40$ sq.cm., $A_L = 0.44$ sq.cm., $V_D = 4.4$ ml.
For chromatogram 5, $V_R = 14.5$ ml., hence K (triol) $= 2.5$
From direct partition, K (triol) $= 3.6$

This solvent system was used in the isolation of a pregnanetriol-like fraction from the urine of normal men (Section 6). In this work non-symmetrical elution curves were obtained on chromatographing bulky urine extracts (5,2d). To overcome this effect the use of 80% aqueous methanol/benzene or benzene-chloroform was considered, and the partition coefficients of 17-hydroxy-11-deoxycorticosterone, 17-hydroxy-11-dehydrocorticosterone and /

FIG. 16. PARTITION CHROMATOGRAMS. (80% MeOH – C_6H_6)



and pregnane-3α:17α:20α-triol in such systems were determined as in the previous experiments.

Results.

TABLE 29. Partition coefficients of pregnane-3α:17α:20α-triol (Triol), 17-hydroxy-11-deoxycorticosterone (S) and 17-hydroxy-11-dehydrocorticosterone (E) in various solvent systems at 17.5 ± 0.5°.

Solvent system	Partition coefficient Triol	S	E
80% methanol/benzene	5.3, 5.3	5.3	7.0
80% methanol/98% benzene-2% chloroform	5.1, 4.8	4.6	5.8
80% methanol/95% benzene-5% chloroform	4.4, 4.0	3.3	5.4
80% methanol/90% benzene-10% chloroform	3.8, 3.4	2.7	5.5

The elution curve obtained with pregnanetriol (100 μg.) using 80% methanol/benzene is shown in Fig. 16. This system was effectively used for the study of compounds in urine extracts as the following experiments show.

L = 10 cm., As = 0.40 sq.cm., A_L = 0.44 sq.cm., V_D = 4.4 ml.
Chromatogram 6, V_R = 20ml., hence K (triol) = 3.9
From direct partition (Table 29), K (triol) = 5.3

5, 4. Partition chromatography of β-glucuronidase-liberated material from n-butanol extracts of urine; solvent system aqueous 80% methanol/benzene.

The chromatographic technique was used to investigate AS and FS liberated by enzymic hydrolysis. The preparation of the urine extracts has been described in detail (4, 9); residues from butanol extracts of the urine of normal men were /

FIG. 17. EFFECT OF SACCHARATE ON THE ENZYMIC HYDROLYSIS OF CONJUGATED 'AS' IN EXTRACTS OF URINE.

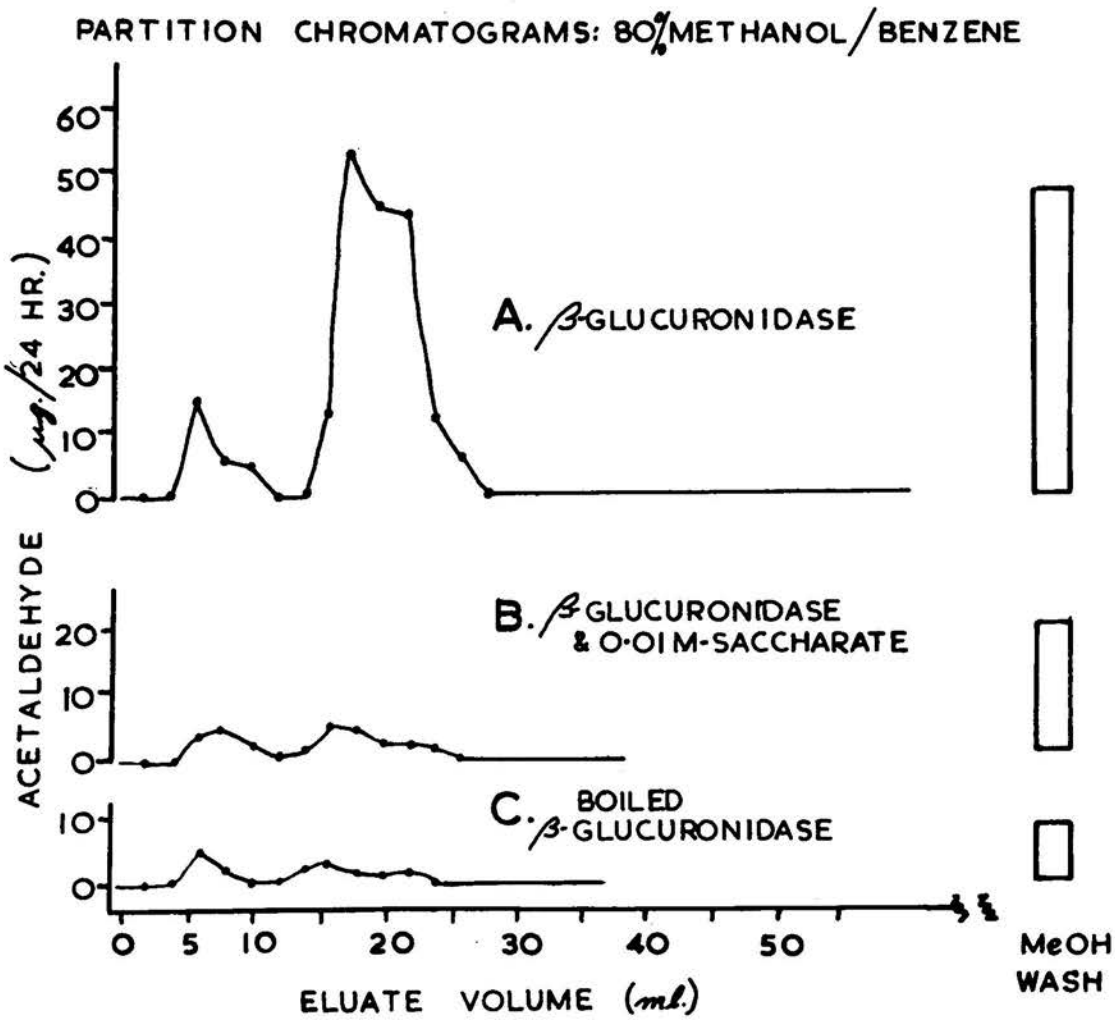
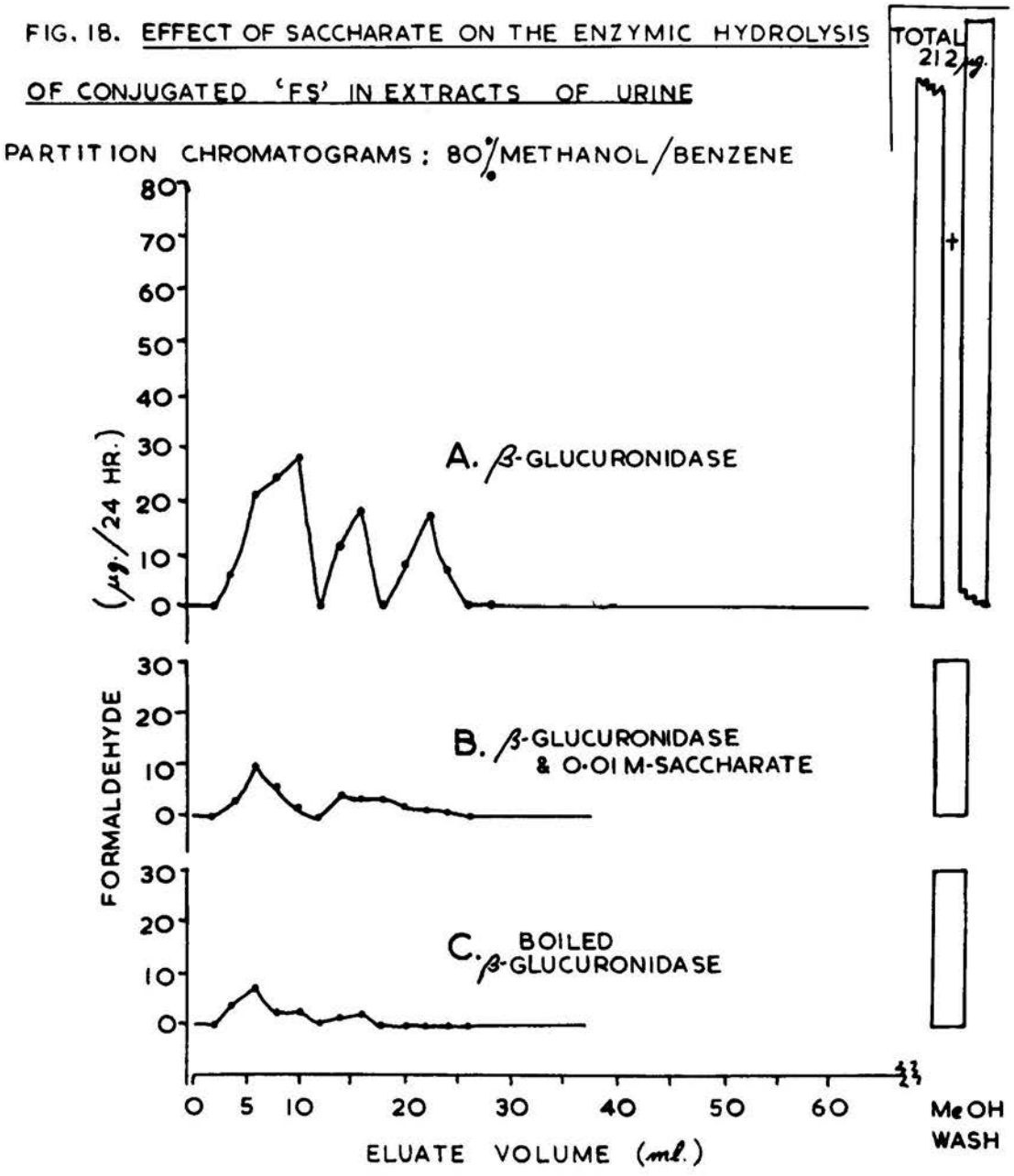


FIG. 18. EFFECT OF SACCHARATE ON THE ENZYMIC HYDROLYSIS OF CONJUGATED 'FS' IN EXTRACTS OF URINE



were incubated with:

(A) β-glucuronidase,
(B) β-glucuronidase + saccharate (0.01 M),
(C) boiled β-glucuronidase.

The chloroform extracts of A, B and C, each obtained from twelve identical thirds of 24 hr. specimens were analysed as in Table 30.

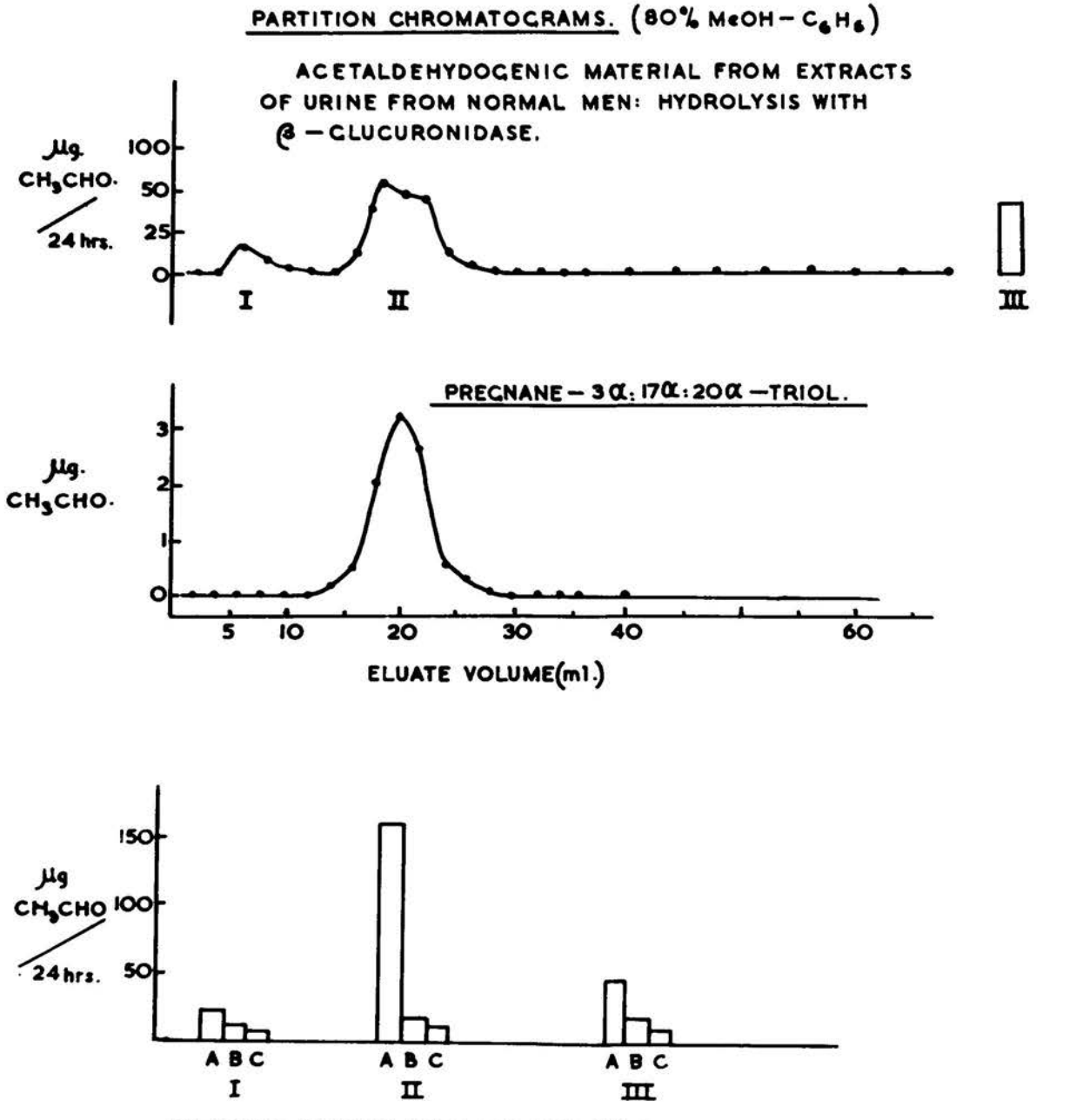
TABLE 30. AS and FS liberated from butanol extracted material from urine by incubation with β-glucuronidase preparations.

Extract incubated with	Acetaldehydogenic substances Acetaldehyde (mg./24 hr.)	Steroid (mg./24hr.)	Formaldehydogenic substances Formaldehyde (mg./24hr.)	Steroid (mg./24hr.)
A. β-glucuronidase	0.284	2.17	0.684	7.53
	0.306	2.34	0.705	7.75
B. β-glucuronidase & saccharate	0.048	0.37	0.068	0.75
	0.049	0.37	0.062	0.68
C. Boiled β-glucuronidase	0.031	0.24	0.045	0.50
	0.037	0.28	0.049	0.54

Portions of these extracts (1.2% A, 6% B, 6% C) were run on partition chromatograms (80% methanol/benzene). The analyses of the eluates are illustrated in Figs. 17 and 18. Material not eluted with mobile phase was washed from the columns with methanol.

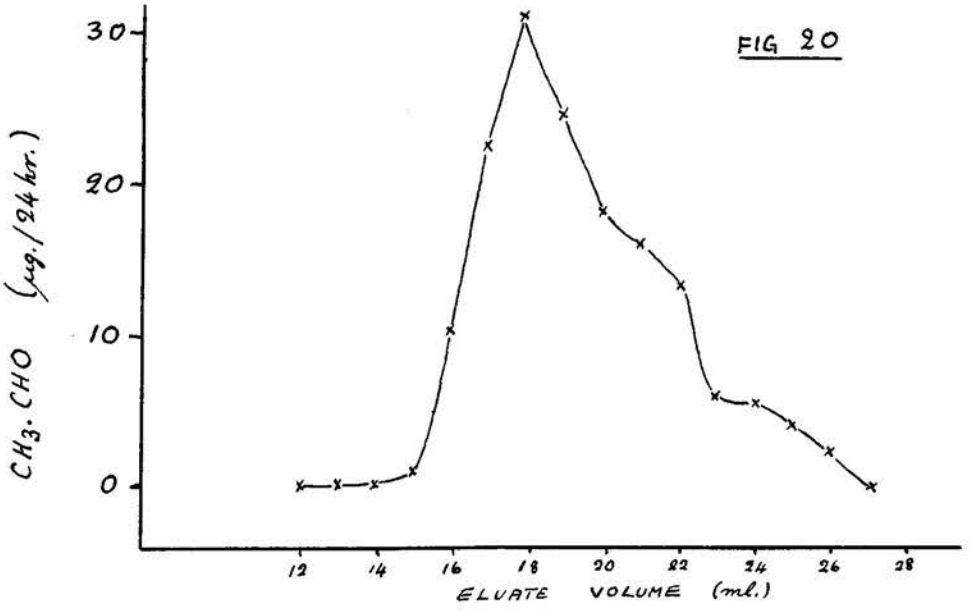
Acetaldehydogenic substances. These fall into three distinct groups: a relatively non-polar fraction (I); a pregnanetriol-like fraction (II) and a polar fraction (III) not eluted by 84 ml. mobile phase ($K > 25$; cf. Table 29). In Fig. 19 the elution of pregnanetriol and of the substances from extract A are compared /

FIG. 19. EFFECT OF SACCHARATE ON THE ENZYMIC HYDROLYSIS OF CONJUGATED ACETALDEHYDOGENIC MATERIAL IN EXTRACTS OF URINE FROM NORMAL MEN



EXTRACTS OF URINE INCUBATED WITH (A) β-GLUCURONIDASE
(B) β-GLUCURONIDASE + SACCHARATE (0·01 M)
(C) BOILED β-GLUCURONIDASE

compared, whilst the block diagram illustrates the effect of enzymic hydrolysis on Groups I, II and III (it was concluded in Section 4, 9 that the difference between extracts A and C was mainly due to the action of β-glucuronidase). β-glucuronidase hydrolysis has the greatest effect on the pregnanetriol-like fraction(II) which is also the largest fraction, accounting for about 70% of the material eluted from the chromatogram. The elution pattern of this fraction seemed to have 2 maxima, so a portion of A (4% A) was run again, and eluate volumes were collected more frequently (Fig. 20).



The result is inconclusive; the lack of symmetry in the elution pattern may have been due to small irregularities in the column.

Formaldehydogenic substances. Some FS appeared in eluates up to about 24 ml. (fraction IV), but the major portion was more polar /

polar material not eluted with 84 ml. mobile phase (fraction V). As the less polar formaldehydogenic material was spread over about 24 ml., often insufficient was present in each eluate portion collected for accurate estimation; no regular pattern of peaks can be discerned. This fraction probably contains $C_{21}O_3$, $C_{21}O_4$ and some $C_{21}O_5$ compounds. β-glucuronidase appears to have a greater effect on the polar fraction V than on fraction IV. This was probably more marked than the results suggest since some of the methanol wash off the column in the case of extract A was lost.

TABLE 31. Effect of saccharate on the enzymic hydrolysis of conjugated FS in extracts of urine

	Amount of steroid obtained (mg./24 hr.)		
	A	B	C
Less polar fractions (IV) (eluate volume 4 - 24 ml.)	1.55	0.38	0.25
Very polar fraction (V) (not eluted with 84 ml. mobile phase)	2.33 [x]	0.33	0.32

[x] small loss during processing

Extracts of urine incubated with:

(A) β-glucuronidase,
(B) β-glucuronidase + saccharate (0.01 M),
(C) boiled β-glucuronidase.

Recovery /

Recovery of material added to column.

From the assays on the individual eluates the following totals were obtained by summation:

TABLE 32. Recovery of AS and FS added to partition columns

Extract	Acetaldehydogenic substances		Formaldehydogenic substances	
	Steroid (mg./24 hr.)	Recovery (%)	Steroid (mg./24 hr.)	Recovery (%)
A	1.85 [x]	82 [x]	3.88 [x]	51 [x]
B	0.36	98	0.71	99
C	0.23	90	0.57	112

[x] Loss during processing of methanol washes of column. These figures however correlate well with the observation that such loss would affect FS more than AS , since a greater proportion of the enzyme hydrolyzed FS are present in the methanol wash fraction (cf. Fig. 18 and Table 31).

5, 5. DISCUSSION.

The solvent systems described give good separations of urinary AS; urinary FS may not be well separated. Recently, Bush (1952) has developed a series of systems for the paper partition chromatography of steroids extracted from blood, using aqueous methanol as the stationary phases and light petroleum, benzene, toluene, ethyl acetate or mixtures of these as the mobile phases. The systems are effective for the separation of compounds of varying polarities from progesterone to some of the components of the 'amorphous fraction' of Wintersteiner & Pfiffner (1936). Bush runs chromatograms at 30 - 38° to reduce tailing, and the use of these high temperatures induces manipulative difficulties; the 'Chromatocoil' invented by Schwarz (1952) provides a simple rapid method of running paper chromatograms at high temperatures.

The agreement between distribution coefficients of pregnanetriol calculated from partition column results, and that obtained by direct solvent partition was not good for the solvent systems 70% methanol/benzene and 80% methanol/benzene (cf. Martin & Synge, 1941; Butt et al. 1951). However, the retention volumes of substances were regular from column to column, and in these and further experiments described in Sections 6 and 7, calculated interrelations of columns of different dimensions were found to be accurate.

Of the three acetaldehydogenic fractions found in the urine extracts, fraction II moved at a rate corresponding to pregnanetriol /

pregnanetriol; as described in Section 6, this fraction was found to contain the latter steroid. The less polar AS if steroidal must have the same C-17 side chain as pregnanetriol, and therefore may differ from it in respect of the 3-hydroxy function. The more polar fraction (III) may contain acetaldehydogenic steroids more highly oxygenated than pregnanetriol.

β-Glucuronidase hydrolysis has notably different effects on AS and FS. Markedly increased amongst the AS is the pregnanetriol-like fraction; amongst the FS, the polar fraction not eluted with mobile phase. This fraction is more polar than 17-hydroxy-11-dehydrocorticosterone. Baggett et al. (1952) report that in the urine of normal adults β-glucuronidase hydrolysis results in a 10 - 50-fold increase in extractable pregnane-3α:17α:21-triol-11:20-dione, but only a 3 - 4-fold increase in extractable pregn-4-ene-17α:21-diol-3:11:20-trione (17-hydroxy-11-dehydrocorticosterone) and pregn-4-ene-11β:17α:21-triol-11:20-dione (17-hydroxycorticosterone).

It may be possible to chromatograph the more polar fractions which were not eluted by the mobile phase in the system 80% methanol/benzene by using a more polar mobile phase. Thus, use of chloroform-benzene mixtures with 80% methanol as 'stationary phase' leads to progressive decrease in the partition coefficients of pregnanetriol, 17-hydroxycorticosterone and 17-hydroxy-11-dehydrocorticosterone as the proportion of chloroform is increased.

SUMMARY./

SUMMARY.

1. Partition chromatographic methods suitable for the purification of steroids, particularly pregnanetriol, in urine extracts have been developed. Hyflo Supercel was used as the supporting phase for aqueous methanol/benzene solvent systems.

2. From partition chromatograms a pregnanetriol-like fraction was found in extracts of urine of normal men.

3. The pregnanetriol-like fraction is increased markedly after β-glucuronidase hydrolysis of urinary material.

4. Acetaldehydogenic substances more and less polar than pregnanetriol are present in urine extracts and are increased after β-glucuronidase hydrolysis.

5. For urinary formaldehydogenic substances, the greatest effect of enzymic hydrolysis is on compounds more polar than 17-hydroxy-11-dehydrocorticosterone.

SECTION 6.

ISOLATION OF PREGNANE-3α:17α:20α-TRIOL FROM THE URINE OF NORMAL MEN.

6, 1. INTRODUCTION

The two urinary 17:20-dihydroxy-20-methyl steroids which have been isolated were obtained from urine samples from patients with hyperfunctioning adrenal glands.

TABLE 34. 17:20-dihyroxy-20-methyl steroids isolated from human urine.

Steroid	Isolated by	Associated condition of patient	Amount isolated (mg./l.)
pregnane-3α:17α:20α-triol[x]	Butler & Marrian 1937	adrenal hyperplasia	40
	Butler & Marrian 1937		16
	Butler & Marrian 1938		14
pregnane-3α:17α:20α-triol[x]	Mason & Kepler (1945)	adrenal hyperplasia	1.5
			1.9
			1.4
		adrenocortical tumour	3.1
pregnane-3α:17α:20-triol	Miller & Dorfman (1950)	adrenal hyperplasia	8
pregn-5-ene-3β:17α:20α-triol	Hirschmann & Hirschmann (1950)	adrenocortical tumour	1.3

Butler & Marrian (1938) also found another triol, $C_{21}H_{36}O_3$, probably pregnane- or allo-pregnane-3β:17:20-triol. It is notable that Butler & Marrian (1937, 1938) and Miller & Dorfman (1950) extracted the compounds from unacidified urine, whereas the other workers used hot acid hydrolyses with probable extensive destruction of these 17-hydroxysteroids. In one case, Mason & Kepler (1945) extracted the urine with butanol and obtained from this extract material precipitated from aqueous solution by acetone as in the method of Venning (1937, 1938) for the isolation of sodium pregnane-3α:20α-diol glucuronidate. The product /

[x] The configuration at C-20 was determined by Klyne (1949).

product which appeared to be a mixture of steroid glucuronides was hydrolyzed with a rat liver enzyme preparation to yield free pregnane-3α:20α-diol and pregnane-3α:17α:20α-triol (1.4 mg./l. urine).

The results of experiments with partition chromatograms (Section 5) indicated that after incubating urine from normal men with β-glucuronidase a triol-like fraction in amounts of the order of 1 mg./24 hr. urine specimen could be obtained. From such a fraction a substance has been isolated and shown to be identical to the pregnane-3α:17α:20α-triol of Butler & Marrian (1937, 1938). This steroid would therefore seem to be a normal constituent of the urine of men.

EXPERIMENTAL

This study was carried out on extracts of urine incubated with β-glucuronidase. These extracts were originally prepared for the enzyme liberated FS and had been stored pending development of suitable chromatographic techniques for the purification and isolation of the steroid components. As the possible number of formaldehydogenic steroids is likely to be greater than the number of acetaldehydogenic steroids in urine, and as several well defined groups of AS appeared to be readily separated on partition chromatograms (Section 5) it was decided to investigate the isolation of the AS first; in particular, compound(s) in the pregnanetriol-like fraction.

6, 2. Preparation of urine extracts.

Urine was collected from male medical students, and pooled batches of 4-10 litres were extracted at pH 2 with chloroform (1 x 2 vol.) to remove free steroids and CSCS (see Section 4). The extracted urine was immediately partially neutralized to pH 5.0 with sodium carbonate solution, and then incubated with β-glucuronidase for 20-24 hr. After cooling to room temperature the mixtures were extracted with chloroform (1 x 0.33 vol., 2 x 0.75 vol.) and the chloroform extracts washed with 0.1 N-NaOH (3 x 0.1 vol.) and water (3 x 0.1 vol.). In some cases the alkali and water washes were saturated with NaCl to reduce loss of steroids. The washed chloroform extracts were dried over the minimum amount of anhydrous sodium sulphate A.R. and the chloroform distilled off under reduced pressure at under 60° /

at under 60°. The residues were dissolved almost completely in warm benzene (20 ml./l. urine extracted) and the benzene solutions stored in the refrigerator. In this way 68 litres of urine were processed. The benzene extracts were then combined, concentrated under reduced pressure to 30 ml. and allowed to stand overnight at 0-5°. The cold benzene extracts were filtered through a sintered glass filter and the residue washed with cold benzene (3 x 7 ml.). There was little benzene insoluble residue. After evaporating the benzene filtrates to dryness, the residue, a red resinous gum (3.07 g.) was dissolved in 100 ml. aqueous 70% ethanol and the solution extracted with n-hexane (4 x 0.6 vol.). The aqueous ethanol phase was concentrated under reduced pressure to remove most of the ethanol and the 20 ml. aqueous solution remaining was extracted with chloroform (4 x 2 vol.). The aqueous mixture was diluted to 100 ml. with water and re-extracted with chloroform (3 x 4 vol.). The combined chloroform extracts were dried and the chloroform distilled off under reduced pressure. The residue, a deep red oil, weighed 1.67 g. and contained the equivalent of 227 mg. 11-deoxycorticosterone compared with 245 mg. found in the combined alkali-washed chloroform extracts before benzene treatment and 70% ethanol-hexane partition.

Further purification of portions of the extract was attempted using benzene-water partition, separation of ketonic and non-ketonic substances with Girard's reagent T (Girard & Sandulesco, 1936) and some chromatographic methods. However, no crystalline /

crystalline compounds were obtained, and the remainder of the extract was stored in the refrigerator.

A study of partition chromatography (Section 5) indicated that the separation of groups of AS and FS in urine extracts and their purification was possible on Supercel columns. It was decided to investigate a possible pregnanetriol-like fraction found in the urine of normal men (Section 5). The procedure used in the processing of the urine extracts described above would be expected to be applicable to AS as well as FS except that the treatment carried out on benzene solutions of the extracted material may have resulted in some loss of pregnanetriol-like steroids; pregnanetriol is sparingly soluble in benzene (Butler & Marrian, 1937).

Estimation of AS in the remaining portion of the urine extracts (0.757 g. of the original 1.670 g.) gave the following values:

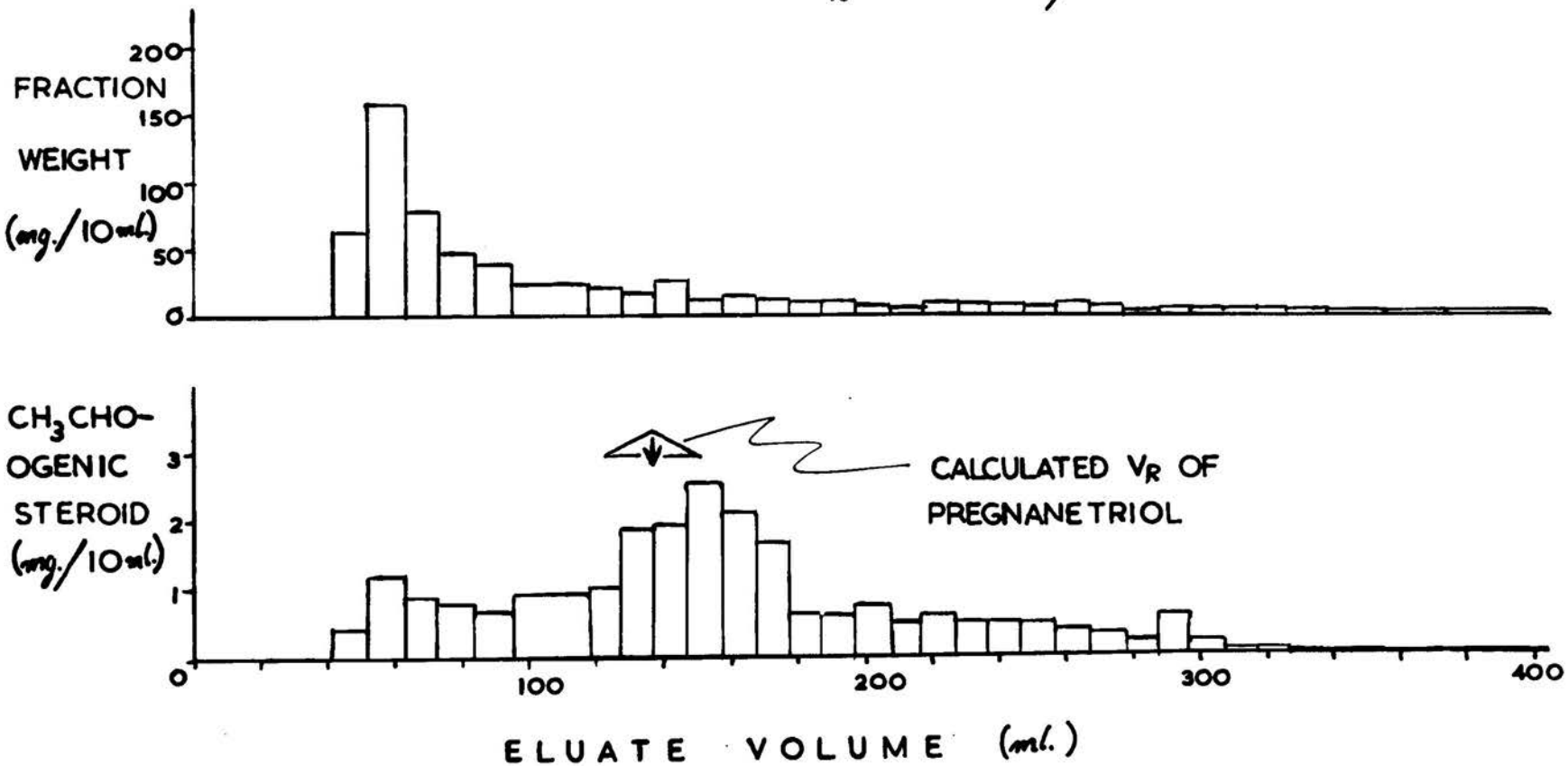
Acetaldehyde found (mg.)	Steroid present (mg.)
6.75	52
6.28	48

6, 3. Partition chromatogram of urine extract; solvent system, aqueous 70% methanol/benzene.

The extract was chromatographed on a Supercel column 35.5 cm. long and 2 cm. in diameter. The extract was transferred to the column in 5 ml. mobile phase. Eluate fractions were collected, usually at 10 ml. intervals, evaporated to dryness on a water bath at 60° under a stream of filtered air, dried over $CaCl_2$ and weighed. The residues were dissolved in 5.0 ml. ethanol /

FIG. 21. PARTITION CHROMATOGRAM OF EXTRACT OBTAINED AFTER INCUBATION OF URINE WITH β-GLUCURONIDASE.

SOLVENT SYSTEM: 70% METHANOL/BENZENE.



ethanol and 0.050 ml. samples withdrawn for estimation of AS. The results are illustrated in Fig. 21. The retention volume of pregnanetriol is shown by an arrow, and was calculated as follows (see Section 5, 2d):

Retention volume of pregnanetriol on chromatogram 5 [x] = 14.5 ml.
Dead volume of chromatogram 5 = 4.4 ml.

Dead volume of above chromatogram = 41 ml.

Hence retention volume of pregnanetriol on above chromatogram $= \frac{41 \times 14.5}{4.4}$

= 135 ml.

The separation of the first two acetaldehydogenic fractions is not complete (Fig. 21). The poor separation and tendency to 'tail' were probably due to too high a concentration of substances for a column of the dimensions used. However, considerable amounts of non-acetaldehydogenic impurities were eluted in the first fractions; the fraction weights demonstrate the purification of the pregnanetriol-like fraction which resulted.

When fractions 7-28 (eluate vol. 95-267 ml.) were re-chromatographed, the same type of pattern was obtained on assaying the AS in the eluates. The weights of the pregnanetriol-like fraction showed little change and thus little purification was effected by the second chromatogram. Attempts were made to crystallize some of the fractions as follows.

6, 4. Isolation /

[x] Chromatogram 5 (Section 5, 3) was carried out on a column 10 cm. long and 1.0 sq. cm. in cross sectional area with the solvent system 70% methanol/benzene.

6, 4. Isolation of pregnanetriol-like substance (Compound X).

Combined eluate fractions, dissolved in ethanol, were filtered through sintered glass filters, and the filtrates evaporated to dryness on a water bath under a rapid stream of filtered air. The residues were moistened with a small drop of methanol and 0.2-0.5 ml. benzene added. The solutions were cooled overnight or for some days in the refrigerator. Any crystals formed were recrystallized from small volumes of benzene-methanol. The crystallizations were, in some cases, repeated. Some crops of crystals were washed with a little ice-cold acetone to remove pigmented material. The melting points of the impure crystals obtained were checked (Table 35).

TABLE 35. Attempted crystallization of fractions from partition chromatogram of urine extract (Fig. 21).

Eluate volume (ml.)	Fraction weight (mg.)	Acetaldehydogenic steroid content (mg.)	(% w/w)	Melting point [x] of impure crystals (°)
118-157	134	9.8	7.3	242-249 246-249 (second crop)
158-197	52	5.1	9.8	241-246
198-257	61	3.6	5.9	no crystals
258-307	37	2.0	5.4	no crystals

The melting points indicated that the three batches of crystals were the same substance, and they were combined for further /

[x] All melting points were determined on a hot-stage apparatus of the Kofler type (Klyne & Rankeillor, 1947), calibrated with pure substances of known melting point.

further purification (total weight, about 6 mg.). Pregnanetriol may be crystallized from methanol (Butler & Marrian, 1937). As so little material was available, it was decided to use aqueous methanol. Pure pregnanetriol was found to have a solubility of 16 mg./ ml. in aqueous 80% methanol. The impure crystals obtained from the urine extract were crystallized twice from about 0.4 ml. 80% methanol, finally yielding 2.3 mg. needle-shaped crystals.

Subsequently, the mother liquors from the above crystallizations and attempted crystallizations (Table 35) were combined. Pregnanetriol was found to be eluted from a magnesium silicate-celite (1:1) adsorption chromatogram (cf. Nelson & Samuels, 1952) with 2-4% ethanol in chloroform. The above mother liquors, dissolved in 5ml. chloroform were chromatographed on 10 g. magnesium silicate-celite (1:1) developing the chromatogram with chloroform and ethanol-chloroform mixtures. Eluates with 2% and 4% ethanol in chloroform were pale coloured oils. These eluates were combined and leached twice with 0.2 ml. cold acetone at about -20°. The almost white solid which remained was crystallized from aqueous ethanol. The crystals, about 5 mg. melted at 243-245°. After two further crystallizations from 80% methanol, fine needle-shaped crystals (2.4 mg.) melting at 246-247° were obtained. This batch of crystals was used for micro-analysis of Carbon and Hydrogen content.

6, 5. Identification /

6, 5. Identification of Compound isolated (Compound X).

Acetylation of pregnanetriol and compound X. Mixtures were made up as follows:

(a) 15 mg. pregnanetriol, 2 ml. acetic anhydride, 0.1 ml. dry pyridine;
(b) about 2 mg. compound X, 0.5 ml. acetic anhydride, 0.05 ml. dry pyridine.

The mixtures were refluxed on a water bath at 100° for 2 hr. Moisture was excluded by the use of $CaCl_2$ tubes. After cooling to 0° the excess acetic anhydride was decomposed with ice-cold water, the mixtures being left at 0° for about 2 hr. The precipitated acetates were filtered through a micro-filter and washed thoroughly with ice-cold water. The acetates were washed through the filter with a little hot ethanol, and after evaporating the ethanol off under reduced pressure, the residues were left overnight in a desiccator over NaOH pellets to remove traces of acetic acid. The residues were crystallized from 50% aqueous ethanol, and then from light petroleum (boiling range 40-60°).

Comparison of pregnane-3α:17α:20α-triol with compound X. Appearance: the two substances and their acetates had the same crystalline appearance after crystallizations from aqueous 80% methanol and aqueous 50% ethanol or light petroleum respectively. Melting points: the observed melting points of pregnanetriol and compound X, and of their acetates as well as mixed melting points are noted in Table 36.

TABLE 36. /

TABLE 36. Melting points

Compound	Melting point (°)
1. Pregnane-3α:17α:20α-triol	247-248
2. Compound X	248-249
3. Mixture of 1 and 2	247-248
4. Pregnane-3α:17α:20α-triol-3:20-diacetate [x]	147-149, 156-159 147-148, 156-158
5. Acetate of Compound X [x]	148-149, 158-159 148-150, 157-159
6. Mixture of 4 and 5 [x]	147-148, 155-157

[x] At the first 'melting point' incomplete fusion with resolidification in changed crystalline form took place. These effects are discussed in the text (see Table 38).

Oxidation with periodic acid: 2.533 mg. Compound X was dissolved in 50.0 ml. ethanol. Portions, 5.00 ml. of this solution were evaporated to dryness and oxidized with periodic acid. Oxidation of known amounts of pregnanetriol were carried out simultaneously.

TABLE 37. Acetaldehyde formed on periodate oxidation of Compound X and pregnane-3α:17α:20α-triol

Compound	Amount oxidized (µg.)	Spekker reading	Acetaldehyde found (µg.)	Steroid as 'pregnanetriol' (µg.)
Pregnanetriol	23.4	0.120	3.21	24.5
	23.4	0.109	2.92	22.3
	23.4	0.111	2.97	22.7
Compound X	25.3	0.126	3.36	25.7
	25.3	0.132	3.53	27.0
	25.3	0.125	3.35	25.6

Analysis: /

Analysis: a further amount of Compound X prepared from the mother liquors enabled a micro-analysis to be carried out.

Analysis of material dried at 60° in vacuo over P_2O_5 for 3 hr.,

2.375 mg. gave 2.271 mg. water and 6.456 mg. carbon dioxide.

Found	C, 74.15;	H, 10.70 %
Calculated for $C_{21}H_{36}O_3$	C, 74.93;	H, 10.79 %

The value obtained for the carbon content is not satisfactory. Drying of the sample (crystallized from aqueous 80% methanol) may not have been complete, or during the crystallization and separation of such small amounts of material a particle of foreign matter may have contaminated the sample.

Conclusion: the melting points and mixed melting points of Compound X and pregnanetriol, and of their acetates together with the quantitative yield of acetaldehyde obtained on periodate oxidation of Compound X, identify the latter as pregnane-3α:17α:20α-triol. The chromatographic position of the band from which Compound X was isolated is consistent with this identification.

Melting points of pregnane-3α:17α:20α-triol-3:20-diacetate.

Difficulty was experienced with the melting points of the acetates of both pregnanetriol and Compound X after crystallization from 50% ethanol. Recrystallization from 50% ethanol did not improve the melting behaviour. The compounds softened at about 136° and appeared to melt with partial re-solidification at about 145°, the crystalline form being different; finally the compounds melted about 155°. Other solvents suitable for the recrystallization of milligram amounts of pregnanetriol-

diacetate /

diacetate were investigated and n-hexane or light petroleum were found to be satisfactory. Recrystallization from light petroleum (boiling range 40-60°) gave fine crystals which after washing and drying melted sharply as shown in Table 36.

The melting point of pregnane-3α:17α:20α-triol-3:20-diacetate quoted by various workers differs (Table 38).

TABLE 38. Reported melting points for pregnane-3α:17α:20α-triol-3:20-diacetate

Authors	Melting point (°)
Butler & Marrian (1937)	136.5
Butler & Marrian (1938)	150-151
Mason & Kepler (1945)	156-158
Klyne (1949), prepared from pregnanetriol of Butler & Marrian (1938);	155-157
prepared from pregnanetriol of Mason & Kepler (1945)	157-159
Author, (acetate prepared from pregnanetriol of Butler & Marrian, 1938)	147-149, 156-158

From the results reported in this Thesis, it would seem that pregnane-3α:17α:20α-triol-3:20-diacetate shows polymorphism, and this may explain the discrepancy between the various melting points reported. Polymorphic behaviour has been reported for pregnane-3α:20α-diol-diacetate (Marrian, 1929; Verly, Sommerville & Marrian, 1950) and for pregn-5-ene-3β-ol-20-one-acetate (Gould & Tarpley, 1951).

6, 6. A further isolation of pregnane-3α:17α:20α-triol from the urine of normal men.

In previous experiments (Sections 4,9 and 5,4) it was shown that the presence of 8×10^{-3} M-saccharic acid resulted in practically complete inhibition of enzymic hydrolysis of urinary conjugated AS; the AS fraction most markedly increased by enzymic action was the pregnanetriol-like fraction. Eluates corresponding to this fraction off a partition chromatogram (80% methanol / benzene; Section 5,4) contained 4.4 mg. as pregnanetriol obtained from the equivalent of 3.6 24 hr. urine specimens from normal male subjects.

The residue from the fraction was leached with a little acetone at about -20°. The almost white solid which remained was crystallized twice from aqueous methanol, and the product washed with a little cold acetone; these crystals, 1.5 mg. melted at 240-245°. A portion, about 0.5 mg., was crystallized twice from acetone-hexane to obtain a melting point. The remainder was acetylated as before (6, 5) and crystallized from 50% aqueous methanol and from light petroleum, boiling range, 40-60°.

Melting /

Melting points.

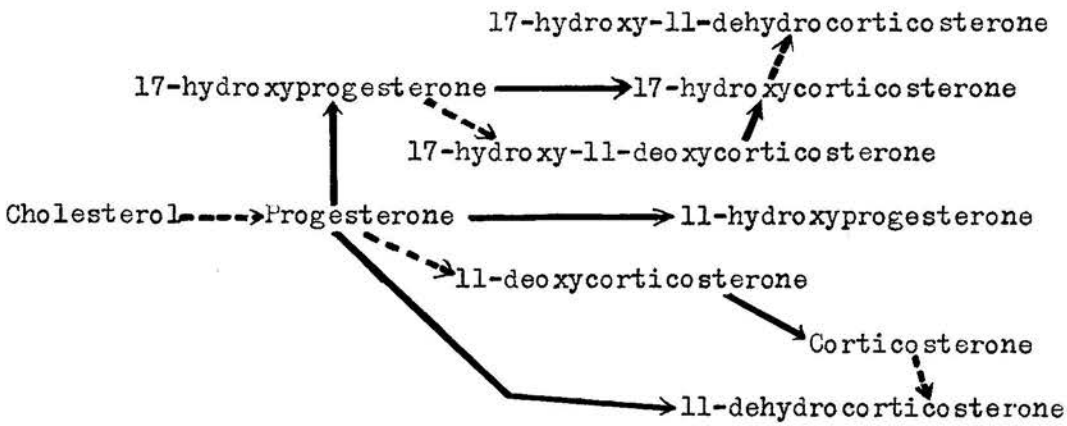
Compound	Melting point (°)
1. Pregnane-3α:17α:20α-triol	246-248
2. Compound X'	245-248
3. Mixture of 1 and 2	245-246
4. Pregnane-3α:17α:20α-triol-3:20-diacetate	145-146, 156-158
5. Acetate of Compound X'	143-146, 156-157
6. Mixture of 4 and 5	145-147, 156-159

It was concluded that the substance isolated was pregnane-3α:17α:20α-triol.

7, 1. INTRODUCTION.

From the metabolic conversions undergone by administered steroids (Section 3, 1) it would appear that 17:20-dihydroxy-20-methylsteroids (21-deoxysteroids) might be reduction products of 21-hydroxysteroids secreted by the adrenal gland. Pincus and co-workers (review: Jacobsen & Pincus, 1951) have shown by perfusion studies with ox-adrenal glands, the probable synthetic pathways for the adrenocortical steroids, in which 17-hydroxycorticosterone and corticosterone are quantitatively the main compounds secreted (Fig. 22).

Fig. 22. Biosynthesis of adrenocortical steroids (after Jacobsen & Pincus, 1951)



⬅⬅⬅➡ conversions not demonstrated, but possible;

⟶ conversions shown to occur.

An increase in urinary acetaldehydogenic steroids after stimulation of the adrenal cortex by administration of adrenocorticotrophic hormone (ACTH) would indicate an adrenal origin /

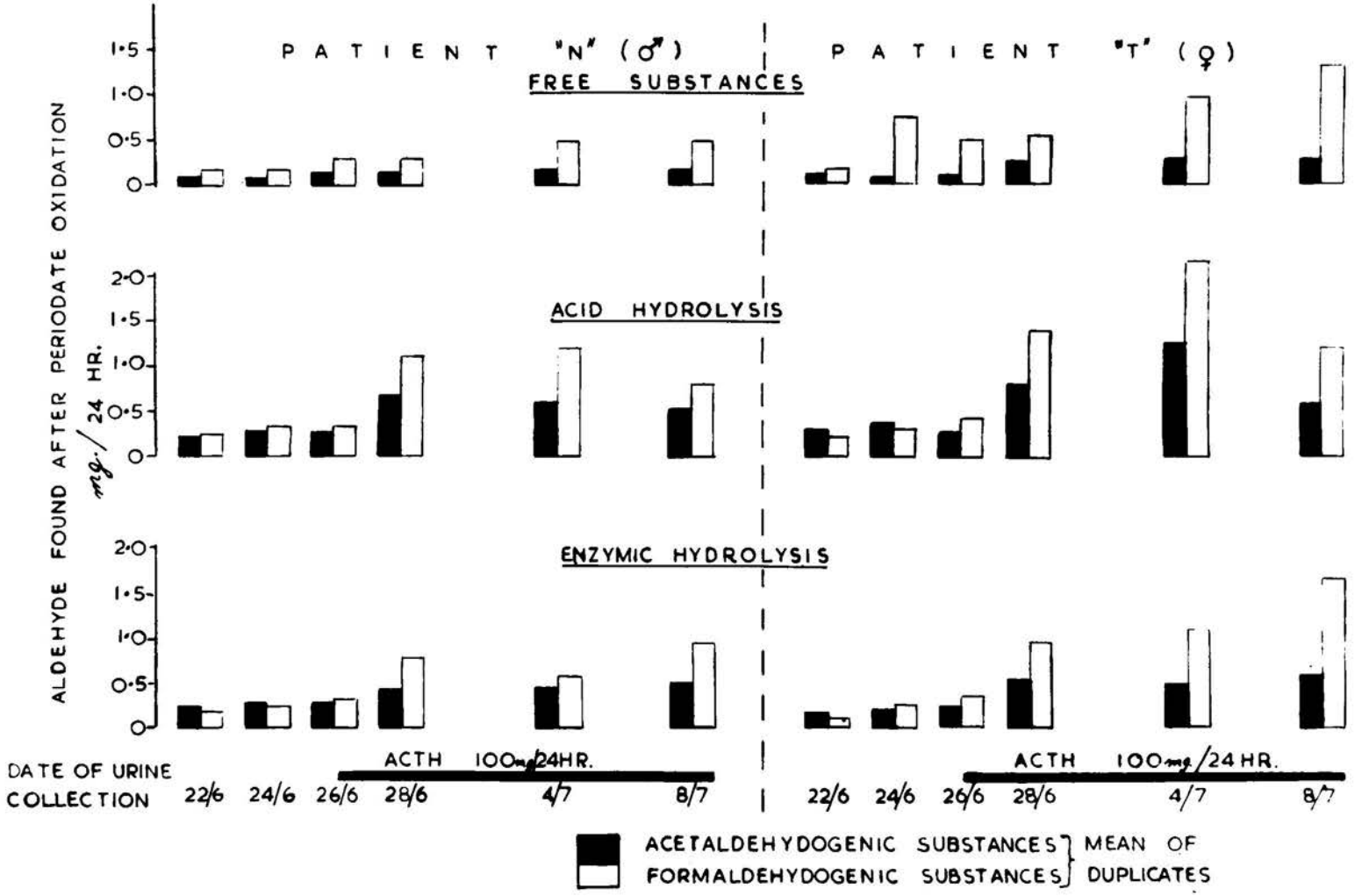
origin for such steroids. An indication as to the precursors of urinary acetaldehydogenic steroids might be obtained by administration of specific steroids to subjects. Pregnane-3α:17α:20α-triol may be formed from 17-hydroxy-11-deoxycorticosterone; it is also conceivable that 17-hydroxyprogesterone is a precursor.

In experiments described in this Section, ACTH administration to 2 patients with rheumatoid arthritis gave some increase in free and conjugated AS in the urine. However, in another case with modified experimental conditions (use of a 0.1 N-NaOH wash of chloroform extracts) neither ACTH nor 17-hydroxy-11-dehydrocorticosterone (cortisone) resulted in a rise in AS in the urine. Thus, the results of these preliminary experiments were inconclusive. Unfortunately there has not been any opportunity up to the present time of repeating these experiments.

In two separate experiments with a normal man, administration of 400 and 606 mg. 17-hydroxy-11-deoxycorticosterone resulted in no significant change in AS in urine. During administration of the steroid, however, large increases in FS indicated that absorption of 17-hydroxy-11-deoxycorticosterone and excretion of it or its formaldehydogenic metabolites had taken place. About 18% of the steroid administered was accounted for by the increase in FS. Partition chromatograms indicated that not only was the total AS unchanged, but that the pregnanetriol-like fraction was not affected by administration of the 17-hydroxy-11-deoxycorticosterone. Enzymic hydrolysis was used in these experiments.

EXPERIMENTAL. /

FIG. 23. EFFECT OF ADMINISTRATION OF ACTH TO PATIENTS SUFFERING FROM RHEUMATOID ARTHRITIS ON EXCRETION OF 'AS' & 'FS' IN URINE



EXPERIMENTAL

7, 2. Administration of adrenocorticotrophic hormone (ACTH) and 17-hydroxy-11-dehydrocorticosterone (cortisone) to patients suffering from rheumatoid arthritis: effect on the urinary AS and FS.

In the first experiment the subjects were N (male) and T (female). Each 24 hr. urine specimen was diluted to 2500 ml. with distilled water. Samples of the diluted urines were chloroform extracted

(a) without treatment (free substances);

(b) after 24 hr. at pH 1.0, room temperature (acid hydrolysis);

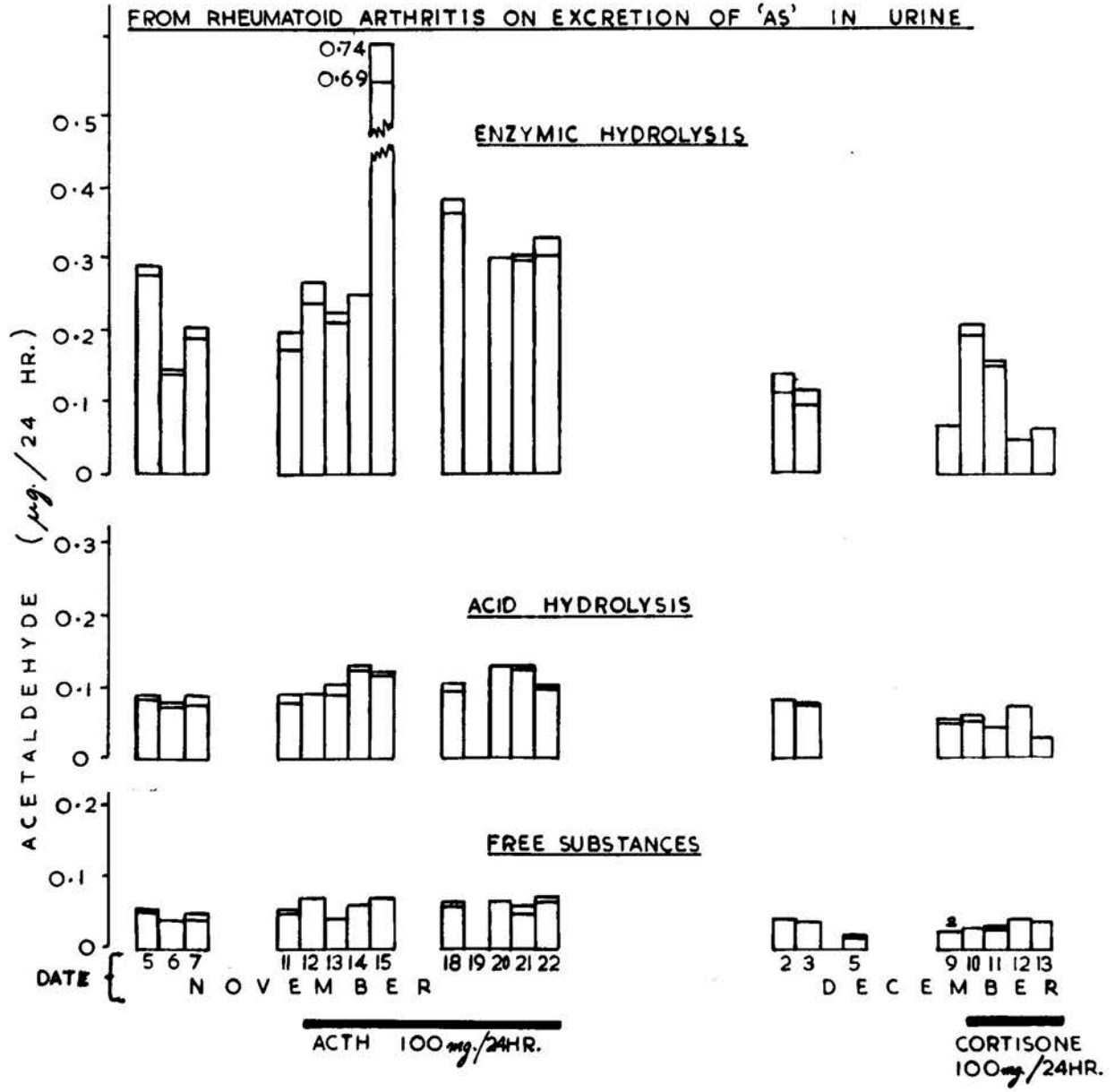
(c) after incubation with β-glucuronidase, 24 hr. at pH 4.5 and 37° (glucuronidase hydrolysis).

The chloroform extracts were not washed with NaOH solutions in this experiment.

Two 24 hr. urine specimens from each patient were examined before ACTH administration and four during administration (100 mg. ACTH / day). The results are illustrated in Fig. 23. During ACTH administration the excretion of free and conjugated AS and FS was increased; FS were generally increased to a relatively greater extent than AS.

The experiment was repeated with a third patient (F, male) who was given first ACTH and later, cortisone (100 mg./day in each case). The estimation of free and conjugated substances was carried out in the same way as before, except that the chloroform extracts were washed with 0.1 N-NaOH and only AS were estimated /

FIG. 24. EFFECT OF ADMINISTRATION OF ACTH AND CORTISONE TO A PATIENT SUFFERING FROM RHEUMATOID ARTHRITIS ON EXCRETION OF 'AS' IN URINE



estimated (Fig. 24). No significant increase was found either for free or conjugated AS during administration of ACTH or cortisone. (It should be noted that pregnanetriol is recovered satisfactorily from urine by chloroform extraction where the chloroform extracts are washed with 0.1 N-NaOH; Section 3, 7). Thus, the results are inconclusive.

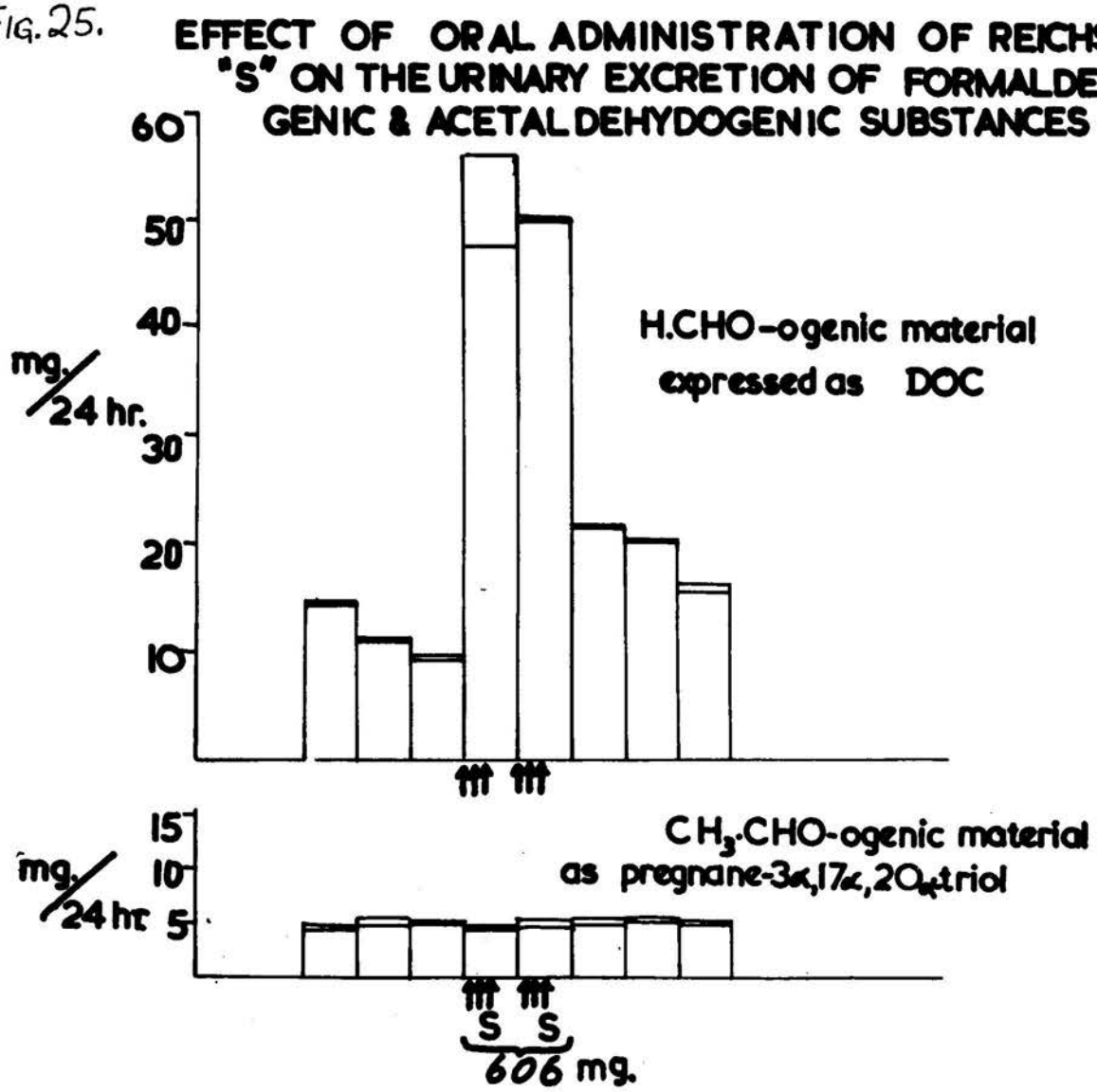
7, 3. Administration of 17-hydroxy-11-deoxycorticosterone (S) to a normal man: effect on the urinary AS and FS liberated by β-glucuronidase hydrolysis

It seemed possible that 17-hydroxy-11-deoxycorticosterone might be a precursor of pregnane-3α:17α:20α-triol isolated from urine (Section 6). The S was administered orally to a normal man and AS and FS levels in urine observed.

Administration of S and collection of urine specimens. Three urine collections were made before administration of the steroid (C1, C2, C3). About 100 mg. (range, 96-105 mg.) of S dissolved in 50 ml. 40% ethanol were taken by the subject together with 20 ml. Rose's Lime Juice three times daily on two consecutive days (at about 8 a.m., 12.30 p.m. and 4.30 p.m.). The urine specimens on these two days and the three subsequent days were collected (S1, S2, S3, S4, S5). Urine specimens were collected for 24 hr. from 9 a.m. over 100 ml. n-butanol.

Processing of urine. The volume of each specimen was adjusted to 2000 ml. and 50 g. sodium bicarbonate was dissolved in it. The diluted urine was extracted with n-butanol (4 x 1 vol.), the butanol extracts were taken to dryness and the residue was dissolved /

Fig. 25.


EFFECT OF ORAL ADMINISTRATION OF REICHSTEIN'S "S" ON THE URINARY EXCRETION OF FORMALDEHYDOGENIC & ACETALDEHYDOGENIC SUBSTANCES
60
50
40
30
20
10
mg./24 hr.
H.CHO-ogenic material expressed as DOC
15
10
5
mg./24 hr
CH3·CHO-ogenic material as pregnane-3α,17α,20α-triol
S S
606 mg.

dissolved in 200 ml. water. Deoxyribose nucleic acid, 0.8 g. in 30 ml. water was added (Bernfeld, Nisselbaum & Fishman, 1952; Brooksbank & Haslewood, 1952) to increase the activity of the enzyme. The mixture was adjusted to pH 4.5, 40 ml. of N-acetate buffer, pH 4.5 were added and the volume was made up to 400 ml. with water. β-Glucuronidase preparation, 100 ml. (290 G.U./ml.) and 20 ml. chloroform were added and the mixture incubated at 37° for 29 hr. After cooling to room temperature, each mixture was extracted with chloroform (3 x 250 ml.). The chloroform extracts were washed with 0.1 N-NaOH (1 x 150 ml.) and water (2 x 150 ml.). Each wash was backwashed with chloroform (100 ml.) which was added to the main chloroform extract before the next wash. The chloroform extracts were dried with about 60 g. anhydrous sodium sulphate A.R. After filtering off the drying agent, the chloroform was distilled off under reduced pressure. The residue was dissolved in 5.0 ml. ethanol and samples of 0.050 ml. withdrawn for the estimation of AS and FS.

The results are shown in Fig. 25. FS were increased markedly, but the levels of AS were not significantly altered. The increase in FS is equivalent to about 108 mg. of 17-hydroxy-11-deoxycorticosterone or 18% of the total administered, and indicates that a reasonable proportion of the steroid was absorbed from the gut. It is noteworthy that the steroid or its formaldehydogenic metabolites were rapidly excreted. Somewhat similar results were obtained in another experiment /

experiment with the same subject when 400 mg. S was administered over two days; in this case enzymic hydrolysis was carried out directly on the urine.

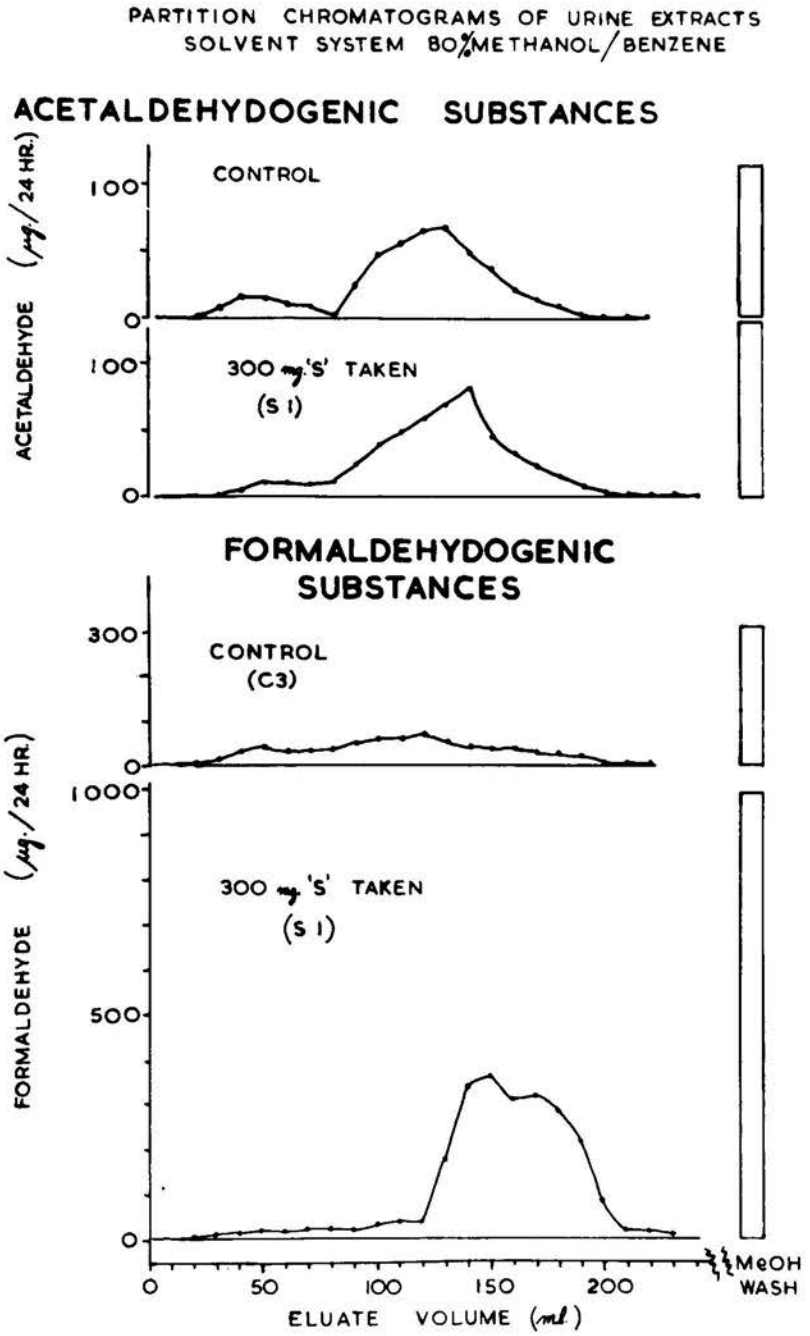
Partition chromatograms of urine extracts obtained before and after administration of 17-hydroxy-11-deoxycorticosterone. Extracts C3 and S1 were used in this study. The residues were dissolved in 21 ml. ethanol and 9 ml. water added; the aqueous 70% ethanol solutions were then extracted with n-hexane (3 x 2 vol.). The hexane was back-washed with 70% ethanol (1 x 0.03 vol.). Hexane solutions were evaporated to dryness. Most of the ethanol was removed from the aqueous ethanol solutions under reduced pressure. After making the volume of the aqueous residues up to 35 ml. with water, 1 g. of NaCl A.R. was added to each and they were extracted with chloroform (4 x 2 vol.). The chloroform extracts, dried over anhydrous sodium sulphate A.R., were evaporated to dryness under reduced pressure. Residues from the hexane and 70% ethanol phases were dried overnight over $CaCl_2$ and weighed.

TABLE 39. Fraction weights after 70% ethanol / hexane partition; loss of AS and FS in hexane phase

Extract	n-Hexane soluble fraction	70% ethanol soluble fraction	AS and FS extracted by n-hexane			
			AS		FS	
	(mg.)	(mg.)	Steroid (mg.)	Loss (%)	Steroid (mg.)	Loss (%)
C3	73	66	0.29	5.7	0.56	5.9
S1	95	183	0.26	5.4	1.42	2.7

The /

FIG. 26. EFFECT OF ADMINISTRATION OF 17-HYDROXY-11-DEOXY-CORTICOSTERONE TO A NORMAL MAN ON THE EXCRETION OF (AS) & (FS) IN URINE (ENZYMIC HYDROLYSIS)



The 70% ethanol soluble fractions were chromatographed on Supercel partition columns 20 cm. long and 2 cm. in diameter (solvent system, 80% methanol / benzene) and the eluate collected in 10 ml. portions. Estimation of AS and FS gave the results illustrated in Fig. 26.

The bands corresponding to the pregnanetriol-like fraction from C3 and S1 contain practically the same amounts of AS. The FS peak which appears in S1 but not in C3 would be consistent with the presence of 17-hydroxy-11-deoxycorticosterone (cf. Table 29, Section 5). The more polar FS is increased notably. Portions of the first 100 ml. eluate indicated a ketosteroid peak at about 50 ml. Ketosteroids were estimated using a modification of the Zimmermann method as recommended by the Medical Research Council Committee on Clinical Endocrinology (1951). This ketosteroid peak was about four-fold as great in S1 as in C3 and may indicate 20- or 17-ketosteroid metabolites of 17-hydroxy-11-deoxycorticosterone. It should be noted that most of the urinary pigments were concentrated in such early eluates and may have rendered these colorimetric estimations unreliable.

It is intended to study further the urine extracts obtained in these experiments with S in an attempt to isolate metabolites of 17-hydroxy-11-deoxycorticosterone. Results of interest have been obtained in initial studies on the main FS band appearing in the chromatogram of urine extract S1 (Fig. 26).

Eluate /

Eluate fractions from 140 - 210 ml. were combined and evaporated to dryness. The residue, a slightly oily semicrystalline white solid (48 mg.) contained the equivalent of 23 mg. Substance 'S'. This residue was purified by crystallization and re-crystallization from various solvents to give stout rod-shaped crystals with the following melting points:

	Melting point of product (°)
1. Crystallized from ethanol-acetone; washed with cold acetone; re-crystallized from ethanol-acetone; crystallized from methanol	187 - 197
2. Crystals from (1) crystallized from acetone-hexane	188 - 200
3. Crystals from (2) crystallized from acetone-hexane (8 mg. crystals)	190 - 202
4. 17-hydroxy-11-deoxycorticosterone crystallized from acetone-hexane	209 - 212

It would appear from the wide range of the 'melting points' of the urinary fraction that the product is impure, but its constancy after crystallization and re-crystallization would seem to indicate a mixture of closely related compounds. The Chromatographic pattern (Fig. 26) is consistent with the presence of two major components in this band of FS. Thus Substance S and closely related formaldehydogenic metabolites may be present in this fraction which is now being examined more closely.

DISCUSSION. /

DISCUSSION.

In considering results of the metabolic experiments described in this Section, it should be remembered that due to the imperfect methods available for the hydrolysis of conjugated AS and FS in urine, only gross differences can be taken as significant. Under the circumstances, all that may be concluded from the experiments in which patients with rheumatoid arthritis were given ACTH is that this treatment is unlikely to have no effect on urinary AS. The possibility that metabolic processes leading to the formation of acetaldehydogenic steroids may not be normal in patients with rheumatoid arthritis must be considered.

The experiments on the administration of 17-hydroxy-11-deoxycorticosterone to a normal man suggest that this steroid is unlikely to be metabolized to a 17:20-dihydroxy-20-methylsteroid, in particular to pregnane-3α:17α:20α-triol. The metabolic precursor of the latter is thus still not indicated. It may be that 17-hydroxyprogesterone, a possible intermediate in the synthesis of adrenocortical steroids is secreted from the adrenal gland and metabolized to pregnane-3α:17α:20α-triol. Further work on this problem is clearly necessary.

SUMMARY

1. During administration of ACTH (100 mg./day) to three patients with rheumatoid arthritis, some increase was found in urinary free and conjugated AS in two cases, but not in the third; in the case of the latter patient, cortisone (100 mg./day) also did not produce any response.

2. In two experiments with a normal man, oral administration of 17-hydroxy-11-deoxycorticosterone (606 mg. and 400 mg.) resulted in no significant alteration in free and β-glucuronidase-hydrolyzable AS in the urine; FS were increased markedly.

3. Partition chromatography of urine extracts obtained before and after administration of 17-hydroxy-11-deoxycorticosterone showed that there was no change in the pregnanetriol-like fraction; formaldehydogenic metabolites of 17-hydroxy-11-deoxycorticosterone were indicated.

APPENDICES.

Appendix I. C_{21} STEROIDS, PROBABLY OF ADRENOCORTICAL ORIGIN, ISOLATED FROM THE URINE OF HUMAN SUBJECTS

The following Tables list the C_{21} steroids of probable adrenal origin obtained from the urine of normal subjects and of patients with hyperactive adrenal glands.

In Table 40 the method by which each steroid was identified is given as 'chromatography' or 'isolation'. In the former case, the identification has been dependent on chromatographic behaviour and spot tests. In the latter case, the steroid has been isolated in a pure crystalline form and characterized by its physical and chemical properties. All the compounds listed in Table 41 were identified after isolation in a pure form.

The method applied to hydrolyze possible steroid conjugates is noted; for acid hydrolysis urines have been acidified to pH 1-2 and allowed to stand for varying lengths of time at room temperature; hot acid hydrolyses have been carried out in strongly acid solution at about 100° for short periods of time; enzymic hydrolyses have been carried out by incubation with crude β-glucuronidase preparations at pH 4-6 at about 37°.

TABLE 40. /

TABLE 40. Steroids, probably of adrenocortical origin, obtained from the urine of normal subjects

Steroid	References	Identification	Hydrolysis applied to urine
allopregnane-3β:11β;17α:21-tetrol-20-one	1952, Romanoff, Wolf & Pincus	Chromatography	Enzymic
pregnane-3α:17α:21-triol-11:20-dione	1950b, 1951, Schneider	Isolation	None
	1952, Schneider	Isolation	Enzymic
	1952, Baggett, Glick & Kinsella	Isolation	Enzymic
allopregnane-3β:17α:21-triol-11:20-dione	1952, Romanoff et al.	Chromatography	Enzymic
pregn-3-ene-11β:17α:21-triol-3:20-dione (17-hydroxycorticosterone)	1951, 1952, Schneider	Isolation	None
	1951, Burton Zaffaroni & Keutman	Chromatography	Acid
	1952, Romanoff et al.	Chromatography	Enzymic
pregnane-17α:21-diol-3:11:20-trione	1951, 1952, Schneider	Isolation	None
allopregnane-17α:21-diol-3:11:20-trione	1952, Romanoff et al.	Chromatography	Enzymic
pregn-3-ene-17α:21-diol-3:11:20-trione (17-hydroxy-11-dehydrocorticosterone)	1950a,b,c, 1951, 1952, Schneider	Isolation	None
	1951, Burton et al.	Chromatography	Acid
	1952, Romanoff et al.	Chromatography	Enzymic
allopregnane-3β:17α:21-triol-20-one	1952, Romanoff et al.	Chromatography	Enzymic
pregnane-3α:20α-diol-11-one	1948a, 1950, Lieberman, Fukushima & Dobriner	Isolation	Hot acid

TABLE 41. Steroids, probably of adrenocortical origin, isolated from the urine of diseased patients

Steroid	References	Disease of patients	Hydrolysis applied to urine or urine extract
21-hydroxysteroids			
pregnane-3α:11β:17α:21-tetrol-20-one	1951, Lieberman, Hariton, Stokem, Studer & Dobriner	Neoplasia [xx]	Acid
pregnane-3α:17α:21-triol-11:20-dione	1950, Lieberman, Hariton & Dobriner	Neoplasia [xx]	Acid and enzymic
pregn-3[4]-ene-11β:17α:21-triol-3:20-dione (17-hydroxycorticosterone)	1948, Mason & Sprague	Adrenal hyperplasia	Acid
	1950, Lieberman et al.	Neoplasia [xx]	Acid and enzymic
	1950, Mason	Post-operative patients; ACTH-treated patients with rheumatoid arthritis	Acid
pregn-3[4]-ene-17α:21-diol-3:11:20-trione (17-hydroxy-11-dehydrocorticosterone)	1951, Lieberman et al.	Neoplasia [xx]	Acid and enzymic
pregn-5-ene-3β:21-diol-20-one	1951, Lieberman et al.	Neoplasia [xx]	Acid and enzymic
pregnan-3α-ol-11:20-dione	1950, Lieberman et al.	Adrenal hyperplasia	Hot acid
21-deoxysteroids(20-methylsteroids)			
pregnane-3α:17α-diol-11:20-dione	1951, Lieberman et al.	Neoplasia [xx]	Acid and enzymic
pregnane-3α:17α:20α-triol	1937, 1938, Butler & Marrian	Adrenal hyperplasia	None
	1945, Mason & Kepler	Adrenal hyperplasia and adrenocortical tumour	Hot acid and enzymic
	1950, Miller & Dorfman	Adrenal hyperplasia	None
pregn-5-ene-3β:17α:20α-triol	1950, Hirschmann & Hirschmann	Adrenocortical tumour	Hot acid
pregnane-3α:20α-diol-11-one	1948a, 1950, Lieberman, Fukushima & Dobriner	Adrenal hyperplasia	Hot acid
pregnane-3α:17α-diol-20-one	1945, Lieberman & Dobriner	Adrenal hyperplasia and adrenocortical tumour	Hot acid
	1947, Mason & Strickler	Female pseudo-hermaphrodite	Acid and enzymic
	1950, Miller & Dorfman	Adrenal hyperplasia	None
	1951, Dobriner, Lieberman, Wilson, Dunham, Sommerville & Rhoads	Rheumatoid arthritis	-
pregn-5-ene-3β:17α-diol-20-one [x]	1947, Hirschmann & Hirschmann	Adrenocortical tumour	Hot acid

[x] Reported also by Lieberman et al. (1948a), but no data as to condition of patient or method of isolation of steroid were given.

[xx] The condition of the subjects was not specified in the original publication; these data are taken from the report of Lieberman & Dobriner (1951).

Appendix II. DEFINITION OF TERMS AND ABBREVIATIONS USED

C_{21} adrenocortical steroid: a steroid characteristic of the cortex of the adrenal gland and having three or more oxygen atoms in the molecule.

Corticoid: a urinary C_{21} steroid of probable adrenocortical origin, having an α-glycol or α-ketol side chain at C-17 and thus on oxidation with periodic acid giving, as one product, formaldehyde.

FS: a urinary substance which on oxidation with periodic acid gives, as one product, formaldehyde; its other properties may provide additional evidence to indicate that it is a corticoid.

AS: a urinary substance which on oxidation with periodic acid gives, as one product, acetaldehyde; its other properties may provide additional evidence to indicate that it is a C_{21} 17:20-dihydroxy-20-methylsteroid.

Pregnanetriol: pregnane-3α:17α:20α-triol.

Acetaldehyde and formaldehyde formed on oxidation of urine extracts with periodic acid, when converted to the equivalent amounts of 'steroid', are arbitrarily expressed as pregnanetriol and 11-deoxycorticosterone respectively.

Polar: the solubility of the adrenocortical steroids in water or aqueous-organic solvents is markedly affected by the substitution in the relatively inert hydrocarbon skeleton of hydroxyl and carbonyl groups which have electrical dipole moments. Hydroxyl groups appear to have a greater effect on solubility than carbonyl groups. The solubility of such steroids in various solvents and their distribution coefficients between two liquid phases are widely used to classify the adrenocortical steroids according to their polarity'. Strictly, the polarity of a substance is a measure of the electrical dissymmetry of the molecule; in the case of the adrenocortical steroids, the latter is probably closely related to their solubility behaviour. Therefore, in the absence of suitable terms, the author has used 'polar' and 'polarity' for adrenocortical steroids according to their solubility in water or aqueous-organic solvents and their distribution between pairs of liquid phases (one being an aqueous phase).

Appendix III. EXPERIMENTAL RESULTS

The numerical results corresponding to Figures given in the text are tabulated in this Appendix; the Figure to which a given Table refers, is noted.

In the assays on eluates of partition chromatograms, Spekker readings smaller than 0.003 are not significant and are denoted by '0' .

TABLE 42. (Fig. 3) Calibration curve using paraldehyde; Barker & Summerson (1941) reaction

Acetaldehyde (µg.)	Spekker reading
3.98	0.521
3.18	0.441
2.39	0.328
1.59	0.212
0.80	0.120

TABLE 43. (Fig. 6) Calibration curves: oxidation of pregnane-3α:17α:20α-triol with periodic acid and estimation of acetaldehyde formed

	Pregnanetriol oxidized (µg.)	Spekker reading
H_2SO_4 bottle 3.9.51	10.0	0.028
	20.1	0.057
	30.2	0.090
	40.2	0.114
	50.2	0.151
	70.3	0.202
H_2SO_4 bottle 24.5.51	14.5	0.079
	17.4	0.096
	29.0	0.170
	43.5	0.248
	58.0	0.328

TABLE 44. (Fig. 9) Effect of pH on the enzymic hydrolysis of urinary conjugated AS and FS

pH of hydrolysis mixtures	Acetaldehydogenic substances		Formaldehydogenic substances	
	Spekker reading	Acetaldehyde (µg./sample)	Spekker reading	Formaldehyde (µg./sample)
3.8	0.049	1.4	0.031	1.4
	0.053	1.5	0.032	1.4
4.5	0.140	3.9	0.074	3.3
	0.130	3.7	0.070	3.1
5.2	0.064	1.8	0.059	2.6
	0.059	1.7	0.056	2.5
5.7	0.068	1.9	0.051	2.3
	0.065	1.8	0.052	2.3
6.2	0.037	1.0	0.066	2.9
	0.041	1.2	0.069	3.1
5.2(blank)	0.011	0.3	0.015	0.7
	0.007	0.2	0.019	0.8

TABLE 45. (Fig. 10) Distribution of β-glucuronidase hydrolyzable conjugated AS and FS in the fractionation of butanol extracts

Fraction			Acetaldehydogenic substances		Formaldehydogenic substances	
			Spekker reading	Steroid (mg./24 hr.)	Spekker reading	Steroid (mg./24 hr.)
I	Extracted urines	A	0.034	0.18	0.059	0.72
			0.020	0.11	0.045	0.55
		B	0.081	0.44	0.169	2.05
			0.077	0.41	0.153	1.86
		C	0.078	0.42	0.169	2.05
			0.085	0.46	0.179	2.18
II	Na_2CO_3 and water washes of butanol extracts	A	0.019	0.10	0.037	0.45
			0.014	0.08	0.044	0.53
		B	0.025	0.13	0.020	0.24
			0.028	0.15	0.018	0.22
		C	0.048	0.26	0.009	0.11
			0.029	0.16	0.013	0.16
III	Chloroform extract of butanol residues	A	0.035	0.19	0.090	1.09
			0.039	0.21	0.065	0.79
		B	0.033	0.18	0.089	1.08
			0.035	0.19	0.093	1.13
		C	0.031	0.17	0.087	1.06
			0.041	0.22	0.085	1.03
IV	Purified butanol extracts	A	0.248	1.33	0.111	1.35
			0.270	1.45	0.111	1.35
		B	0.189	1.02	0.112	1.36
			0.204	1.10	0.109	1.32
		C	0.181	0.97	0.107	1.30
			0.190	1.02	0.080	0.97
V	Unextracted urine		0.150	0.81	0.158	1.92
			0.151	0.81	0.163	1.98

TABLE 46. (Fig. 11) Acetaldehydogenic and formaldehydogenic substances liberated from butanol extracts of urine by incubation with (A) β-glucuronidase, (B) β-glucuronidase saccharate (0.01 M) and (C) boiled β-glucuronidase

Urine	incubation	Acetaldehydogenic substances		Formaldehydogenic substances	
		Acetaldehyde (mg./24hr.)	Steroid (mg./24hr.)	Formaldehyde (mg./24hr.)	Steroid (mg./24hr.)
I	A	0.436	3.33	0.849	9.33
		0.444	3.36	0.743	8.16
	B	0.085	0.65	0.146	1.61
		0.070	0.53	0.141	1.55
	C	0.057	0.43	0.106	1.16
		0.056	0.42	0.103	1.13
III	A	0.482	3.67	0.690	7.56
		0.485	3.70	0.672	7.38
	B	0.077	0.58	0.101	1.11
		0.089	0.67	0.109	1.20
	C	0.092	0.70	0.093	1.02
		0.089	0.67	0.104	1.13
	D	0.013	0.10	0.048	0.53
	enzyme blank	0.010	0.08	0.032	0.35
IV	A	0.422	3.23	0.846	9.30
		0.443	3.38	0.786	8.65
	B	0.076	0.58	0.139	1.53
		0.063	0.49	0.149	1.64
	C	0.069	0.53	0.099	1.10
		0.069	0.53	0.116	1.28
	D	0.013	0.10	0.060	0.66
	enzyme blank	0.025	0.19	0.053	0.59
VI	A	0.253	1.94	0.401	4.41
		0.245	1.87	0.451	4.94
	B	0.025	0.19	0.066	0.73
		0.032	0.24	0.060	0.65
	C	0.021	0.16	0.020	0.21
		0.023	0.18	0.035	0.38
	D	0.000	0.00	0.023	0.25
	enzyme blank	0.008	0.06	0.007	0.08
VIII	A	0.319	2.44	0.922	10.13
		0.335	2.56	0.942	10.35
	B	0.061	0.47	0.192	2.11
		0.057	0.44	0.202	2.22
	C	0.044	0.34	0.129	1.42
		0.042	0.32	0.123	1.35
	D	0.034	0.26	0.083	0.91
	enzyme blank	0.027	0.21	0.070	0.76

Note: analyses on totals I - XII A, B, C are given in Section 4, 9.

TABLE 47. Chromatograms of pregnanetriol and a urine extract; solvent system aqueous 50% methanol/67% benzene-33% hexane
(Fig. 13)

	Total eluate volume (ml.)	Spekker reading	Acetaldehyde (μg.)	Steroid (μg.)
I. Pregnanetriol	2.5	0	-	-
	4.0	0	-	-
	5.5	0	-	-
	7.0	0.105	2.95	22.6
	8.5	0.073	2.05	15.7
	10.0	0.020	0.56	4.3
	11.5	0	-	-
	13.0	0	-	-
	14.5	0	-	-
	16.0	0	-	-
	17.5	0	-	-
	19.0	0	-	-
II. Pregnanetriol	3.5	0	-	-
	5.5	0	-	-
	7.2	0.050	1.41	10.8
	8.9	0.040	1.12	8.6
	10.6	0.010	0.28	2.2
	12.3	0	-	-
	14.0	0	-	-
	15.7	0	-	-
	17.4	0	-	-
III. Urine extract	1.7	0	-	-
	3.4	0	-	-
	4.9	0	-	-
	5.9	0	-	-
	6.9	0.010	0.28	2.2
	7.9	0.020	0.56	4.3
	8.9	0.030	0.84	6.5
	9.9	0.010	0.28	2.2
	10.9	0	-	-

TABLE 48. (Fig. 15) Partition chromatograms of pregnanetriol (75 µg.); solvent system, aqueous 70% methanol/ benzene

	Fraction volume (ml.)	Total eluate volume (ml.)	Spekker reading	Pregnanetriol found (µg.)
I.	10.0	10	0	-
	2.0	12	0.020	4.3
	3.0	15	0.250	53.7
	3.0	18	0.051	11.0
	2.0	20	0.008	1.7
	4.0	24	0	-
	2.0	26	0	-
	3.0	29	0	-
	3.0	32	0	-

Amount of pregnanetriol recovered, 70.7 µg. or 94% recovery.

II.	10.0	10	0	-
	1.0	11	0	-
	1.0	12	0	-
	0.5	12.5	0	-
	0.5	13	0.012	2.6
	0.5	13.5	0.030	6.5
	0.5	14	0.051	11.0
	0.5	14.5	0.069	14.8
	1.0	15.5	0.059	12.7
	1.5	17	0.088	18.9
	1.25	18.25	0.021	4.5
	1.25	19.5	0	-
	1.25	20.75	0	-
	3.0	23.75	0	-
	14.0	37.75	0	-

Amount of pregnanetriol recovered, 71.0 µg. or 95% recovery.

TABLE 49. (Fig. 16) Partition chromatogram of pregnanetriol; solvent system, 80% methanol / benzene

Total eluate volume (ml.)	Spekker reading	Acetaldehyde found (µg.)	Pregnanetriol found (µg.)
0-8 (discarded)	-	-	-
10	0	-	-
12	0	-	-
14	0.007	0.20	1.5
16	0.030	0.84	6.4
18	0.110	3.07	23.6
20	0.180	5.05	38.7
22	0.150	4.22	32.2
24	0.028	0.79	6.0
26	0.012	0.34	2.6
28	0	-	-
30	0	-	-

TABLE 50. (Fig. 17 & 18) Partition chromatogram of extract A (urinary substances after incubation with β-glucuronidase); solvent system, 80% methanol / benzene

Total eluate volume (ml.)	Acetaldehydogenic substances			Formaldehydogenic substances		
	Spekker reading	Acetaldehyde (μg./24hr.)	Steroid (μg./24hr)	Spekker reading	Formaldehyde (μg./24hr.)	Steroid (μg./24hr)
2	0	-	-	0	-	-
4	0	-	-	0.006	5.5	60
6	0.025	14.6	110	0.023	21.2	250
8	0.009	5.3	40	0.027	24.9	270
10	0.008	4.7	40	0.031	28.5	310
12	0	-	-	0	-	-
14	0	-	-	0.012	11.0	120
16	0.021	12.3	90	0.020	18.4	200
18	0.090	52.6	400	0	-	-
20	0.076	44.5	340	0.008	7.4	80
22	0.074	43.3	330	0.019	17.5	190
24	0.020	11.7	80	0.007	6.4	70
26	0.009	5.3	40	0	-	-
28	0	-	-	0	-	-

Subsequent fractions up to 84 ml. gave negligible Spekker readings; the material remaining on the column was washed off with methanol:

Methanol wash	0.080	46.8	360	0.230	212	2330

TABLE 51. (Fig. 17 & 18) Partition chromatogram of extract B (urinary substances after incubation with β-glucuronidase in the presence of 8×10^{-3} M-saccharic acid); solvent system, 80% methanol / benzene

	Acetaldehydogenic substances			Formaldehydogenic substances		
Total eluate volume (ml.)	Spekker reading	Acetaldehyde (μg./24hr.)	Steroid (μg./24hr)	Spekker reading	Formaldehyde (μg./24hr.)	Steroid (μg./24hr)
2	0	-	-	0	-	-
4	0	-	-	0.015	2.8	30
6	0.033	3.9	30	0.052	9.6	105
8	0.037	4.3	33	0.029	5.3	59
10	0.020	2.3	18	0.010	1.8	20
12	0.004	0.5	4	0	-	-
14	0.010	1.2	9	0.022	4.1	45
16	0.046	5.4	41	0.020	3.7	40
18	0.038	4.5	34	0.017	3.1	34
20	0.024	2.8	21	0.011	2.0	22
22	0.018	2.1	16	0.009	1.6	18
24	0.012	1.4	11	0.004	0.7	8
26	0	-	-	0	-	-

Subsequent fractions up to 36 ml. gave negligible Spekker readings; the material remaining on the column was washed off with methanol:

Methanol wash	0.165	19.3	147	0.163	30.0	330

TABLE 52. (Fig. 17 & 18) Partition chromatogram of extract C (urinary substances after incubation with boiled β-glucuronidase); solvent system, 80% methanol / benzene

	Acetaldehydogenic substances			Formaldehydogenic substances		
Total eluate volume (ml.)	Spekker reading	Acetaldehyde (µg./24hr.)	Steroid (µg./24hr)	Spekker reading	Formaldehyde (µg./24hr.)	Steroid (µg./24hr)
2	0	-	-	0	-	-
4	0.007	0.8	6	0.022	4.1	45
6	0.045	5.3	40	0.040	7.4	81
8	0.021	2.5	19	0.014	2.6	28
10	0.007	0.8	6	0.016	3.0	32
12	0.007	0.8	6	0.005	0.9	10
14	0.020	2.3	18	0.010	1.8	20
16	0.030	3.5	27	0.012	2.2	24
18	0.014	1.6	12	0.004	0.7	8
20	0.014	1.6	12	0	-	-
22	0.015	1.8	13	0	-	-
24	0	-	-	0	-	-

Subsequent fractions up to 36 ml. gave negligible Spekker readings; the material remaining on the column was washed off with methanol:

Methanol wash	0.074	8.7	66	0.158	29.2	320

TABLE 53. (Fig. 20) Partition chromatogram of the pregnanetriol-like fraction of extract A (urinary substances after incubation with β-glucuronidase); solvent system, 80% methanol / benzene

Total eluate volume (ml.)	Acetaldehydogenic substances		
	Spekker reading	Acetaldehyde (μg./24 hr.)	Steroid (μg./24 hr.)
0 - 11 (discarded)	-	-	-
12	0	-	-
13	0	-	-
14	0	-	-
15	0.005	1.0	7
16	0.052	10.2	75
17	0.115	22.5	166
18	0.159	31.0	230
19	0.126	24.6	182
20	0.092	18.0	133
21	0.082	16.0	119
22	0.068	13.3	98
23	0.031	6.0	45
24	0.028	5.5	40
25	0.021	4.1	30
26	0.010	2.0	15
27	0	-	-

TABLE 54. (Fig. 21) Partition chromatogram of urine extract obtained after β-glucuronidase hydrolysis; solvent system, 70% methanol/benzene

Fraction	Fraction volume (ml.)	Residue appearance	Residue weight (mg.)	Acetaldehydogenic steroid/fraction (mg.)
1	41	Trace of material	-	-
2	10	Red oil	61	0.43
3	11	Red oil	172	1.31
4	11	Red semi-solid oil	86	0.97
5	11	" " " "	52	0.88
6	11	" " " "	42	0.75
7	22	" " " "	53	2.15
8	10	Yellow semi-solid oil	24	1.09
9	10	" " " "	17	1.91
10	10	" " " "	27	1.96
11	10	" " " "	13	2.60
12	10	Pale semi-solid oil	15	2.15
13	10	Red oil	14	1.70
14	10	" "	11	0.63
15	10	" "	12	0.66
16	10	" "	10	0.77
17	10	" "	9	0.55
18	10	Yellow oil	12	0.66
19	10	" "	12	0.56
20	10	" "	10	0.54
21	10	" "	8	0.53
22	10	Yellow semi-solid oil	11	0.42
23	10	" " " "	9	0.39
24	10	" " " "	5	0.28
25	10	" " " "	5	0.65
26	10	" " " "	7	0.28
27	10	" " " "	7	0.15
28	10	" " " "	7	0.17
29	13	" " " "	8	0.11
30	15	" " " "	9	0.17
31	14	" " " "	5	0.11
32	27	" " " "	9	0.22
33	175	Red semi-solid oil	38	0.71

TABLE 55. (Fig. 23) Effect of administration of ACTH to a patient (N, male) suffering from rheumatoid arthritis on AS and FS in urine

Urine collection and volume (ml.)	Hydrolysis used	Acetaldehydogenic substances		Formaldehydogenic substances	
		Acetaldehyde (mg./24hr)	Steroid (mg./24hr)	Formaldehyde (mg./24hr)	Steroid (mg./24hr)
22/6, 1150	None	0.094	0.72	0.149	1.64
		0.104	0.80	0.168	1.85
	Acid	0.225	1.72	0.224	2.47
		0.244	1.86	0.240	2.65
	Enzymic	0.208	1.60	0.158	1.74
		0.254	1.94	0.166	1.82
24/6, 1900	None	0.094	0.72	0.168	1.85
		0.108	0.83	0.146	1.61
	Acid	0.256	1.96	0.327	3.60
		0.264	2.01	0.327	3.60
	Enzymic	0.259	1.98	-	-
		0.294	2.24	0.227	2.50
26/6, 1800 →	None	0.110	0.84	0.265	2.92
		0.083	0.63	0.282	3.10
	Acid	0.238	1.82	0.326	3.60
		0.268	2.05	0.325	3.57
	Enzymic	0.268	2.05	0.330	3.63
		0.300	2.30	0.279	3.06
28/6, 1200 →	None	0.115	0.88	0.251	2.77
		0.119	0.91	0.300	3.30
	Acid	0.680	5.20	1.12	12.3
		0.647	4.95	1.10	12.1
	Enzymic	0.441	3.38	0.751	8.30
		0.409	3.12	0.765	8.41
4/7, 1900 →	None	0.165	1.23	0.475	5.21
		0.172	1.32	0.466	5.11
	Acid	0.597	4.56	1.19	13.1
		0.561	4.31	1.20	13.2
	Enzymic	0.393	3.00	0.571	6.30
		0.444	3.39	0.590	6.50

Table 55 (continued) /

Table 55 (continued)

Urine collection and volume (ml.)	Hydrolysis used	Acetaldehydogenic substances		Formaldehydogenic substances	
		Acetaldehyde (mg./24hr)	Steroid (mg./24hr)	Formaldehyde (mg./24hr)	Steroid (mg./24hr)
8/7, 1900 →	None	0.165	1.23	0.500	5.50
		0.172	1.32	0.449	4.94
	Acid	0.477	3.65	0.831	9.18
		0.551	4.24	0.751	8.29
	Enzymic	0.509	3.88	0.951	10.4
		0.460	3.52	0.930	10.2

TABLE 56. (Fig. 23) Effect of administration of ACTH to a patient (T, female) suffering from rheumatoid arthritis on AS and FS in urine

Urine collection and volume (ml.)	Hydrolysis used	Acetaldehydogenic substances		Formaldehydogenic substances	
		Acetaldehyde (mg./24hr)	Steroid (mg./24hr)	Formaldehyde (mg./24hr)	Steroid (mg./24hr)
22/6, 2720	None	0.124	0.95	0.177	1.95
		0.106	0.81	0.176	1.93
	Acid	0.314	2.40	0.211	2.32
		0.286	2.18	0.239	2.64
	Enzymic	0.145	1.11	0.124	1.36
		0.157	1.20	0.156	1.72
24/6, 2200	None	0.081	0.62	0.729	0.80
		0.060	0.46	0.742	0.82
	Acid	0.357	2.72	0.313	3.45
		0.379	2.90	0.295	3.25
	Enzymic	0.204	1.56	0.268	2.95
		0.211	1.61	0.233	2.56

Table 56 (continued) /

Table 56 (continued)

Urine collection and volume (ml.)	Hydrolysis used	Acetaldehydogenic substances		Formaldehydogenic substances	
		Acetaldehyde (mg./24hr)	Steroid (mg./24hr)	Formaldehyde (mg./24hr)	Steroid (mg./24hr)
26/6, 2200 →	None	0.067	0.51	0.521	5.75
		0.068	0.52	0.406	4.46
	Acid	0.257	2.05	0.409	4.50
		0.281	2.16	0.419	4.61
	Enzymic	0.222	1.70	0.357	3.94
		0.251	1.92	0.346	3.81
28/6, 1900 →	None	0.233	1.78	0.552	6.10
		0.198	1.52	0.474	5.20
	Acid	0.769	5.88	1.38	15.2
		0.764	5.85	1.39	15.3
	Enzymic	0.553	4.24	0.919	10.1
		0.511	3.91	0.980	10.8
4/7, 3800 →	None	0.263	2.01	0.927	10.2
		0.288	2.20	0.944	10.4
	Acid	1.26	9.62	2.13	23.3
		1.23	9.34	2.17	23.9
	Enzymic	0.511	3.91	1.13	12.4
		0.565	4.34	1.11	12.2
8/7, 2500 →	None	0.243	1.86	1.34	14.8
		0.247	1.89	1.25	13.8
	Acid	0.551	4.30	1.28	14.0
		0.630	4.84	1.11	12.2
	Enzymic	0.589	4.50	1.69	18.6
		0.535	4.10	1.64	18.0

→ administration of ACTH, 100 mg./day from 26/6 to 8/7, to each patient (Tables 55 and 56).

TABLE 57. (Fig. 24) Administration of ACTH and cortisone to a patient suffering from rheumatoid arthritis: effect on excretion of AS in urine

Hydrolysis used	Urine collection and volume (ml.)	Acetaldehydogenic substances Acetaldehyde (mg./24hr)	Steroid (mg./24hr)	Urine collection and volume (ml.)	Acetaldehydogenic substances Acetaldehyde (mg./24hr)	Steroid (mg./24hr)
None	5/11	0.056	0.43	13/11 →	0.041	0.31
	2250	0.054	0.41	805	0.042	0.32
Acid		0.094	0.72		0.090	0.69
		0.088	0.67		0.106	0.81
Enzymic		0.282	2.16		0.225	1.72
		0.266	2.04		0.207	1.58
None	6/11	0.039	0.30	14/11 →	0.055	0.42
	1220	0.039	0.30	1090	0.054	0.41
Acid		0.082	0.63		0.134	1.02
		0.076	0.58		0.121	0.94
Enzymic		0.138	1.06		0.244	1.86
		0.136	1.04		0.251	1.92
None	7/11	0.045	0.34	15/11 →	0.065	0.50
	1750	0.047	0.37	1630	0.071	0.54
Acid		0.092	0.70		0.120	0.92
		0.078	0.60		0.124	0.95
Enzymic		0.200	1.53		0.691	5.30
		0.186	1.42		0.734	5.61
None	11/11	0.056	0.43	18/11 →	0.060	0.46
	2000	0.046	0.35	2300	0.065	0.50
Acid		0.078	0.60		0.101	0.77
		0.092	0.70		0.106	0.81
Enzymic		0.199	1.52		0.366	2.80
		0.178	1.36		0.352	2.68
None	12/11 →	0.067	0.51	20/11 →	0.062	0.51
	1480	0.071	0.54	2150	-	-
Acid		0.092	0.70		0.128	0.98
		0.090	0.69		-	-
Enzymic		0.265	2.02		0.296	2.26
		0.230	1.75		-	-

Table 57 (continued) /

Table 57 (continued)

Hydrolysis used	Urine collection and volume (ml.)	Acetaldehydogenic substances Acetaldehyde (mg./24hr)	Steroid (mg./24hr)	Urine collection and volume (ml.)	Acetaldehydogenic substances Acetaldehyde (mg./24hr)	Steroid (mg./24hr)
None	21/11 → 2070	0.047	0.36	9/12 1150	0.025	0.19
		0.060	0.46		0.024	0.18
Acid		0.129	0.99		0.056	0.43
		0.126	0.96		0.051	0.39
Enzymic		0.304	2.32		0.069	0.53
		0.288	2.20		-	-
None	22/11 → 2300	0.072	0.55	10/12 ⟹ 2240	0.030	0.23
		0.063	0.48		0.030	0.23
Acid		0.101	0.77		0.051	0.39
		0.106	0.81		0.059	0.45
Enzymic		0.301	2.30		0.207	1.58
		0.320	2.45		0.191	1.46
None	2/12 2450	0.044	0.34	11/12 ⟹ 2000	0.027	0.21
		0.044	0.34		0.033	0.25
Acid		0.089	0.68		0.042	0.32
		0.081	0.62		0.042	0.32
Enzymic		0.116	0.89		0.149	1.14
		0.144	1.10		0.154	1.18
None	3/12 2000	0.039	0.30	12/12 ⟹ 1460	0.039	0.30
		0.039	0.30		-	-
Acid		0.073	0.56		0.072	0.55
		0.075	0.57		-	-
Enzymic		0.098	0.75		0.046	0.35
		0.114	0.87		-	-
None	5/12 1160	0.012	0.09	13/12 ⟹ 1000	0.035	0.27
		0.018	0.14		-	-
Acid		-	-		0.031	0.24
		-	-		-	-
Enzymic		-	-		0.061	0.47
		-	-		-	-

→ administration of ACTH, 100 mg./day 12/11 to 22/11

⟹ administration of cortisone, 100 mg./day 10/12 to 13/12

TABLE 58. (Fig. 25) Effect of administration of 606 mg. 17-hydroxy-11-deoxycorticosterone (S) to a normal man on the excretion of AS and FS in urine

Urine sample		Urine volume (ml.)	Extract weight (mg.)	Acetaldehydogenic substances		Formaldehydogenic substances	
				$CH_3.CHO$ (mg./24hr)	Steroid (mg./24hr)	H.CHO (mg./24hr)	Steroid (mg./24hr)
25/5	(C1)	1200	180	0.61	4.71	1.28	14.1
				0.63	4.84	1.32	14.6
26/5	(C2)	1450	169	0.72	5.52	1.02	11.2
				0.67	5.11	1.01	11.1
27/5	(C3)	1260	163	0.67	5.11	0.84	9.20
				0.66	5.01	0.89	9.77
28/5	(S1)	1460	268	0.61	4.71	5.11	56.2
				0.63	4.85	4.33	47.6
29/5	(S2)	1170	261	0.67	5.14	4.56	50.1
				0.63	4.79	4.57	50.3
30/5	(S3)	1400	191	0.65	4.99	1.98	21.9
				0.69	5.30	1.96	21.6
31/5	(S4)	1800	200	0.69	5.31	1.85	20.4
				0.68	5.21	1.86	20.5
1/6	(S5)	1860	225	0.68	5.19	1.50	16.5
				0.65	4.97	1.44	15.8

→ 17-hydroxy-11-deoxycorticosterone administered, about 300 mg./day (Section 7, 3).

TABLE 59. (Fig. 26) Partition chromatogram of urine extract (enzymic hydrolysis) during administration of 17-hydroxy-11-deoxycorticosterone to a normal man; extract S1.

Fraction volume (ml.)	Acetaldehydogenic substances		Formaldehydogenic substances	
	Acetaldehyde (µg./24hr)	Steroid (mg./24hr)	Formaldehyde (µg./24hr)	Steroid (mg./24hr)
30	0	0	0	0
10	3	0.02	8	0.09
10	6	0.05	5	0.06
10	11	0.08	13	0.14
10	11	0.08	10	0.11
10	10	0.08	14	0.15
10	12	0.09	15	0.17
10	25	0.19	14	0.15
10	39	0.30	22	0.24
10	47	0.36	31	0.34
10	58	0.45	30	0.33
10	67	0.51	168	1.85
10	80	0.61	332	3.65
10	42	0.32	354	3.89
10	31	0.24	309	3.40
10	22	0.17	314	3.45
10	14	0.11	283	3.11
10	6	0.05	213	2.34
10	3	0.02	85	0.93
10	2	0.01	17	0.19
10	1	0.01	14	0.15
10	2	0.01	9	0.10
Methanol wash	131	1.00	989	10.9

TABLE 60. (Fig. 26) Partition chromatogram of urine extract (enzymic hydrolysis) before administration of 17-hydroxy-11-deoxycorticosterone to a normal man; extract C3.

Fraction volume (ml.)	Acetaldehydogenic substances		Formaldehydogenic substances	
	Acetaldehyde (µg./24hr)	Steroid (mg./24hr)	Formaldehyde (µg./24hr)	Steroid (mg./24hr)
30	0	0	0	0
10	6	0.05	8	0.09
10	16	0.12	27	0.30
10	15	0.12	33	0.36
10	10	0.07	24	0.27
10	9	0.07	24	0.27
10	3	0.03	29	0.32
10	24	0.18	46	0.51
10	48	0.36	52	0.57
10	54	0.41	53	0.58
10	64	0.49	60	0.66
10	67	0.51	48	0.52
10	47	0.36	38	0.41
10	36	0.28	35	0.39
10	20	0.15	33	0.36
10	14	0.11	27	0.30
10	8	0.06	26	0.29
10	3	0.02	19	0.20
10	1	0.01	4	0.05
10	2	0.01	3	0.03
10	1	0.01	2	0.02
Methanol wash	113	0.86	312	3.42

Appendix IV. PURIFICATION OF ORGANIC SOLVENTS

Most of the methods used are described by Weissberger & Proskauer (1935). Fractionating columns generally used in distillations were 40 cm. long and packed with glass beads.

Acetic acid, aldehyde free: acetic acid A.R. was refluxed with chromium trioxide for 4 hr. and then distilled through a fractionating column. Potassium bichromate A.R. has been found as effective as chromium trioxide; the presence of small amounts of water was not a disadvantage.

Acetone: commercially pure acetone was refluxed with potassium permanganate (1 g./l.) for 6 hr. If necessary more potassium permanganate was added. After distillation the acetone was dried over potassium carbonate and fractionally distilled.

Benzene: benzene A.R. was dried over sodium wire, decanted off and distilled through a fractionating column.

n-Butanol: after refluxing with NaOH (5 g./l.) for 6 hr. the butanol was distilled twice through a fractionating column.

Chloroform: B.P. grade was dried with anhydrous sodium sulphate A.R., distilled and stored in dark bottles away from sunlight.

Diethyl ether: the A.R. grade was shaken with ferrous sulphate in/

in H_2SO_4 (600 g. $FeSO_4.7H_2O$, 60 ml. concentrated H_2SO_4, 1100 ml. water), with N-NaOH solution and then with water until the washings were neutral. After drying over anhydrous calcium chloride, the ether was distilled and stored in dark bottles.

Ethanol: after refluxing with NaOH (5 g./l.) the ethanol was distilled off and redistilled through a fractionating column.

Ethanol, aldehyde free: ethanol, refluxed with and distilled off NaOH was shaken with m-phenylenediamine (2 g./l.) and allowed to stand at least a week in the dark. The ethanol was then distilled as required through a small fractionating column.

n-Hexane: 'aromatic free' grade hexane was washed with concentrated H_2SO_4 A.R. (50 ml./l.) until the washings were no longer coloured. The hexane was then washed with water, dried over sodium carbonate and sodium wire and fractionally distilled.

Methanol: methanol was refluxed for 6 hr. with NaOH (5 g./l.) and doubly distilled through a fractionating column.

Appendix V. REFERENCES

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PUBLICATIONS.

1. "Some Observations on the Adreno-cortical Steroids in Human Urine" (with J.Y.F.Paterson & G.F.Marrian), Biochem. J., 1950, 46, xxix.

2. "The Hydrolysis of the Chloroform-insoluble Conjugated Adrenocortical Steroids in Human Urine" (with G.F.Marrian), Biochem. J., 1951, 48, xxxiii.

3. "A Method for the Quantitative Determination in Urinary Extracts of C_{21} 17:20-Dihydroxy-20-methylsteroids", Biochem. J., 1952, 52, 339.

Biochem. J., 1950, Vol. 46, p. xxix.]

Some Observations on the Adreno-cortical Steroids in Human Urine. By J. Y. F. Paterson, R. I. Cox and G. F. Marrian. (*Department of Biochemistry, University of Edinburgh*)

There are two general types of method for the estimation of urinary adrenal cortical steroids. One type of method estimates these steroids by the reducing power of suitably prepared extracts of urine (Talbot, Saltzman, Wixom & Wolfe, 1945; Heard & Sobel, 1946; Heard, Sobel & Venning, 1946). The other type of method estimates these steroids by the formaldehyde generated on oxidation of urine extracts with periodic acid (Lowenstein, Corcoran & Page, 1946; Daughaday, Jaffe & Williams, 1948). By either type of method the amount of adreno-cortical steroids which can be extracted from urine is increased by preliminary acidification of the urine. This suggests that these steroids may be excreted, at least in part, as conjugates.

In the present work the conditions necessary for the optimum hydrolysis of these presumed conjugates were studied, using a slightly simplified Daughaday method. Urine was acidified to pH 1, allowed to stand at 25°, and duplicate aliquots withdrawn for estimation at various time intervals. Estimations were also done on aliquots of the urine which had not been acidified. The hydrolysis curve so obtained was unexpected (Fig. 1, solid points), and indicates that the formaldehydogenic substances estimated by this method may be divided into two groups. The first group is acid labile, and the other stable to acid under the conditions used.

Heard *et al.* (1946) have observed that when urine is acidified, the amount of 'reducing substance' which can be extracted by chloroform-ether is increased. If, before extraction, the urine is neutralized immediately after acidification, the amount of reducing substance extracted is nearly the same as that extracted from unacidified urine. In the present work, hydrolysis curves, in which one of the duplicate aliquots was neutralized before extraction, were done. The results (see figure) confirm Heard's observations and indicate that both groups of formaldehydogenic substances are conjugated. The conjugates of the first (labile) group can be extracted by chloroform from acid urine, but not from neutral urine. The conjugates of the second (stable) group cannot be extracted by chloroform either from neutral or acid urine.

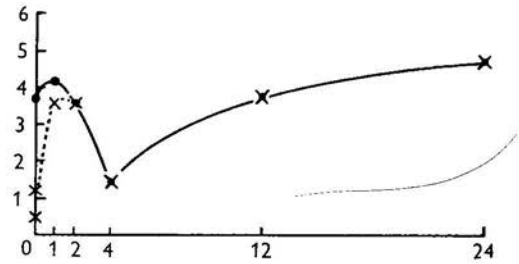

Fig. 1. Hydrolysis curve: normal male. Abscissa: time interval between acidification and extraction (solid points) or acidification and neutralization (followed immediately by extraction) (crosses) in hours. Ordinate: formaldehyde (μg.) per aliquot (1/15 of 24-hr. urine).

Increases in the amounts of both groups of substances have been found in pregnancy, in a case of Cushing's syndrome, and on administration of ACTH to a rheumatoid arthritic woman. Two abnormal hydrolysis curves have been observed.

The importance of the above results in routine estimations is stressed. Talbot uses extracts of unacidified urine; Heard *et al.*, Lowenstein *et al.* and Daughaday *et al.* extract immediately after acidification, while Mason & Sprague (1948) allow the urine to hydrolyse for 1–3 days. All of these are obviously not 'optimum hydrolysis conditions'.

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Biochem. J., 1951, Vol. 48, p. xxxiii.]

The Hydrolysis of the Chloroform-insoluble Conjugated Adrenocortical Steroids in Human Urine. By R. I. Cox and G. F. Marrian. (*Department of Biochemistry, University of Edinburgh*)

Concentrates containing part of the conjugated formaldehydogenic steroids not extractable from acidified urine by chloroform (see preceding communication) were prepared in the following manner: after extraction of urine with chloroform to remove free steroids and chloroform-soluble conjugates, the acidified urine was extracted with *n*-butanol. The conjugated formaldehydogenic steroids were then extracted from the *n*-butanol by shaking with aqueous NaOH.

Incubation of such extracts at 37° and pH 5 with crude preparations of ox-spleen β-glucuronidase yielded free formaldehydogenic steroid in amounts about ten times greater than could be obtained by acid hydrolysis for 24 hr. at pH 1 and 37°.

Greatly increased yields of formaldehydogenic steroid have also been obtained by incubation of urine at pH 5 with β-glucuronidase after removal of unconjugated steroid and chloroform-soluble conjugated steroid by preliminary chloroform extraction at pH 1. Somewhat similar results have been noted by Kinsella, Doisy & Glick (1950) with an enzyme preparation from *Escherichia coli*, using the Heard-Sobel procedure for determining 'reducing' steroids.

The free formaldehydogenic steroid fraction obtained from the chloroform-insoluble conjugated compounds by the action of β-glucuronidase appears to contain two different types of compound, one of which is slowly destroyed at pH 1 and 37°, the other being relatively stable under these conditions.

This destruction by acid would explain the observation (Pincus & Romanoff, 1950) that continuous extraction with ethylene dichloride during acid hydrolysis at pH 1 yields greater amounts of 'reducing' steroid from urine than are obtainable by extraction after hydrolysis.

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A Method for the Quantitative Determination in Urinary Extracts of C_{21} 17:20-Dihydroxy-20-methylsteroids

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Hyperactivity of the adrenal cortex in human subjects due to malignant neoplasm, hyperplasia or stimulation by adrenocorticotropic hormone (ACTH) has frequently been observed to be associated with the urinary excretion of considerable amounts of various C_{21} 20-methylsteroids (see Table 1).

Table 1. *20-Methylsteroids, probably of adrenal origin, isolated from human urine*

Compound	Associated condition of subjects	References
Pregnane-3α:17α:20α-triol	Adrenal hyperplasia	Butler & Marrian (1937, 1938)
	Adrenal hyperplasia	Mason & Kepler (1945)
	Adrenocortical tumour	
Pregnane-3α:17α-diol-20-one	Adrenal hyperplasia and adrenocortical tumour	Lieberman & Dobriner (1945)
	Adrenal hyperplasia	Miller & Dorfman (1950)
Pregn-5-ene-3β:17α:20α-triol	Adrenocortical tumour	Hirschmann & Hirschmann (1950)
Pregn-5-ene-3β:17α-diol-20-one	Adrenocortical tumour	Hirschmann & Hirschmann (1947)
Pregnane-3α:20α-diol-11-one	Adrenal hyperplasia	Lieberman, Fukushima & Dobriner (1950)
Pregnan-3α-ol-11:20-dione	Adrenal hyperplasia	Lieberman *et al.* (1950)
Pregnane-3α:17α-diol-11:20-dione	After administration of ACTH and cortisone to patients with neoplasia	Lieberman *et al.* (1951)

Such urinary 20-methylsteroids may arise directly from related 20-methylsteroids present in the adrenal glands, but there is good reason to suppose that they may in part arise by metabolic reduction at C-21 of 21-hydroxysteroids secreted by the glands. The evidence that such metabolic reduction of 21-hydroxysteroids to 20-methylsteroids, i.e. to 21-deoxysteroids can occur in the human body appears to be conclusive. Thus Cuyler, Ashley & Hamblen (1940) and Horwitt, Dorfman, Shipley & Fish (1944) isolated pregnane-3α:20α-diol from the urine of human subjects after the administration of 11-deoxycorticosterone acetate; Mason (1948) isolated pregnane-3α:20α-diol-11-one after the administration of 11-dehydrocorticosterone; while more recently Lieberman, Hariton, Stockem, Studer & Dobriner (1951) have isolated pregnane-3α:17α-diol-11:20-dione after administering cortisone acetate. It has been suggested that the principal C_{21} adrenocortical hormone secreted by the adrenal glands in man may be 17-hydroxycorticosterone (Reich, Nelson & Zaffaroni, 1950; Hechter, 1950; Pincus, Hechter & Zaffaroni, 1951). In view of this possibility and because of the widespread therapeutic use of 17-hydroxy-11-dehydrocorticosterone (cortisone), it was thought that a specific method for the quantitative determination in urine of C_{21} 17:20-dihydroxy-20-methylsteroids which might be formed from these two hormones (and from 17-hydroxy-11-deoxycorticosterone) by metabolic reduction of the C-21 hydroxyl group, would be of some value and interest.

As is well known, C_{21} steroids with either α-ketol or α-glycol side chains give almost quantitative yields of formaldehyde on oxidation with periodic acid, and this reaction has been widely employed for the quantitative determination in suitably prepared urine extracts of the so-called 'formaldehydogenic' steroids (Lowenstein, Corcoran & Page, 1946; Corcoran & Page, 1948; Daughaday, Jaffe & Williams, 1948). On oxidation with periodic acid, C_{21} 17:20-dihydroxy-20-methylsteroids, and steroids of no other type, should yield acetaldehyde. Accordingly, the possibility was envisaged of determining both formaldehydogenic and acetaldehydogenic steroids in urinary extracts by periodate oxidation with separation and estimation of the formaldehyde and acetaldehyde thus produced. Talbot & Eitingon (1944) have estimated the 17-ketosteroids formed after periodate oxidation of urine extracts, thus measuring the combined total of C_{21} 17:20:21-trihydroxysteroids and C_{21} 17:20-dihydroxy-20-methylsteroids.

With pregnane-3α:17α:20α-triol (Butler & Marrian, 1937, 1938) preliminary experiments showed that nearly quantitative yields of acetaldehyde as determined colorimetrically in sulphuric acid with 4-hydroxydiphenyl (Miller & Muntz, 1938; Koenemann, 1940; Barker & Summerson, 1941) could be obtained after oxidation with periodic acid. In further experiments in which mixtures of pregnane-3α:17α:20α-triol and 11-deoxycorticosterone or 17-hydroxy-11-deoxycorticosterone were oxidized with periodic acid, it was found possible to remove the acetaldehyde quantitatively from a reaction mixture containing glycine without loss of formaldehyde, by aeration into a bisulphite trap (cf. Shinn & Nicolet, 1941). The formaldehyde could then be recovered quantitatively in the usual way by distillation (Daughaday *et al.* 1948).

To test out the potentialities of the procedure for the determination of acetaldehydogenic steroids in urine, recovery experiments were carried out in which known amounts of pregnane-3α:17α:20α-triol were added to both unwashed and sodium hydroxide-washed chloroform extracts of urine from normal men. These extracts were obtained from fresh urine, from urine after incubation with ox-spleen β-glucuronidase at pH 4·5, and from urine extracted after standing at room temperature at pH 1 for 24 hr. In every experiment 'blank' determinations were carried out on a portion of the extract with no added pregnanetriol.

Reasonably satisfactory recoveries were obtained in those experiments in which the triol was added to sodium hydroxide-washed chloroform extracts of urine. In those in which unwashed extracts were employed, the recoveries were irregular, indicating possibly the presence of material interfering in some way with the periodate oxidation of the triol. It may be noted that Hollander, Di Mauro & Pearson (1951), studying the recovery in periodate oxidations of 11-deoxycorticosterone added to urine extracts, concluded that the oxidation could be inhibited by substances present in crude extracts.

The 'blank' experiments all indicated the presence of acetaldehydogenic material in the extracts, and it is noteworthy that from the various extracts the amounts of acetaldehyde obtained were roughly of the same order as those of formaldehyde and, like the latter, were increased both by glucuronidase and acid hydrolysis of the urine. It is also noteworthy that there was little or no increase in chloroform extractable acetaldehydogenic material over that obtained from untreated urine when incubation with glucuronidase was carried out in the presence of saccharate. Since the latter is a competitive inhibitor of β-glucuronidase (Karunairatnam & Levvy, 1949), this supports the suggestion that acetaldehydogenic material released during enzymic incubation was present as a β-glucuronide. It is pertinent to note that Mason & Kepler (1945) isolated from adrenal carcinoma urine a mixture of steroid conjugates which yielded pregnane-3α:17α:20α-triol after incubation with a crude rat-liver glucuronidase preparation.

As far as can be judged from recovery experiments with pregnane-3α:17α:20α-triol, the procedure developed should provide a satisfactory method for determining acetaldehydogenic steroids in sodium hydroxide-washed chloroform extracts of untreated urine, and of urine hydrolysed enzymically or with acid. It is not claimed that this procedure provides a means for the quantitative determination of such steroids in urine, since no demonstrably quantitative methods have yet been developed for the hydrolysis of the conjugated acetaldehydogenic steroids which must be assumed to be present in urine, or for the extraction of the free steroids after hydrolysis. In fact, in these respects the method is neither better nor worse than the widely employed procedures for the determination of urinary reducing or formaldehydogenic steroids, the difficulties involved in which have been recently summarized by Marrian (1951).

EXPERIMENTAL

Apparatus. The apparatus used is shown in Fig. 1. *A* is the oxidation tube; *B* contains aqueous $NaHSO_3$ solution in which acetaldehyde, carried over from the oxidation mixture in the air stream, is trapped. A second bisulphite trap has not been found necessary under the conditions used.

Pregnane-3α:17α:20α:-triol, m.p. 245–247° (Butler & Marrian, 1938) has been used throughout as the reference acetaldehydogenic steroid. All steroid samples were dried *in vacuo* over P_2O_5 before weighing. Standard solutions were prepared in redistilled ethanol.

Method. Ethanolic solutions of pure steroids or of suitably prepared urine extracts are evaporated in oxidation tubes *A* (Fig. 1) to about 1 ml. under a rapid stream of filtered air on a water bath at about 80°. Evaporation to dryness is carried out *in vacuo* with warming on a water bath. The residues are moistened with 0·10 ml. glacial acetic acid (distilled off CrO_3). The tubes are warmed slightly in a water bath and rotated to wet any residue adhering to the sides of the tube. Equal volumes of 0·12 M-periodic acid in 0·4 N-H_2SO_4 (A.R.) and 1 % glycine in 0·4 N-H_2SO_4 (A.R.) are freshly mixed, and 1 ml. portions of the mixture pipetted into the oxidation tubes. The trap tubes *B*, each containing 2 ml. 1 % $NaHSO_3$ solution, are immediately connected up. A slow stream of air, purified by passage through conc. H_2SO_4 (A.R.) and a soda-lime tower is blown through the sets of tubes arranged in parallel. The rate of aeration is about 5 ml./min.

After 45 min. of oxidation with aeration, the air flow is stopped and the tubes are disconnected. The formaldehyde retained in the oxidation mixture is estimated as in the method of Daughaday *et al.* (1948), using 3 ml. 6 % (w/v) $SnCl_2$ (A.R.) solution to reduce the correspondingly larger excess of periodic acid present.

The bisulphite traps are made up to 3 ml. by washing down the ends of the connecting tubes with distilled water,

and 1 ml. samples of the solutions pipetted into dry 150 × 25 mm. test tubes. The colorimetric estimation of acetaldehyde in these samples is then carried out according to Barker & Summerson (1941), except that 7·0 ml. portions of conc. H_2SO_4 (A.R.) are added to the samples, and colour development is carried out at 25°.

The absorption of the solutions is determined with a Hilger Spekker absorptiometer using Ilford no. 605 filters.

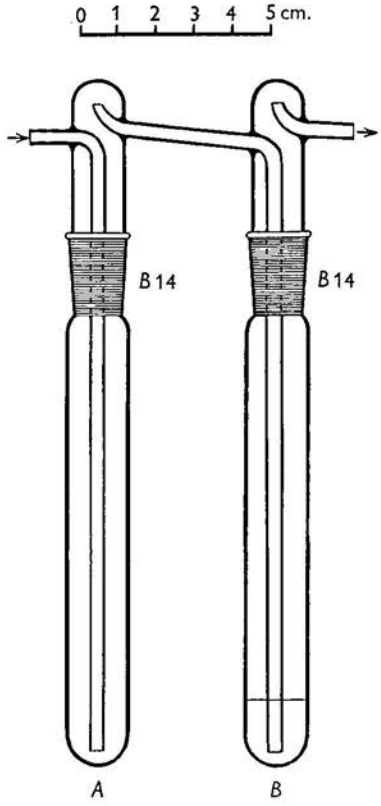

Fig. 1. Apparatus used for oxidation with aeration. *A*, oxidation tube; *B*, bisulphite trap graduated at 3·0 ml. Arrow indicates direction of air stream.

Colorimetric estimation of acetaldehyde

The method of Miller & Muntz (1938), as modified by Koenemann (1940) and Barker & Summerson (1941), must be rigorously standardized to obtain reproducible results. Particular attention should be paid to the purity of the A.R. H_2SO_4. For instance, Russell (1944) has shown that presence of nitrates or nitrites in the sulphuric acid affects the colour development. Therefore each bottle of H_2SO_4 should be checked before use and those giving poor colour development discarded.

Also to be noted is the advice of Barker & Summerson (1941) against the use of chromic acid mixtures for cleaning apparatus used in the acetaldehyde estimations, because of the difficulty of removing interfering traces of the cleaning agent.

Optimum conditions for the oxidation of pregnane-3α:17α:20α-triol

The effects of varying periodic acid concentration, time of aeration and acidity of the oxidation mixture on the yield of acetaldehyde obtained by periodate oxidation of pregnanetriol are illustrated in Tables 2–4. Apart from the variable studied, other conditions in these experiments were as described in the method. The oxidation-aerations have been carried out at room temperature, i.e. 13–21° and within this range the recoveries of acetaldehyde have not been affected by temperature.

Table 2. *Effect of periodic acid concentration on oxidation of pregnane-3α:17α:20α-triol*

(58·0 μg. pregnanetriol samples oxidized.)

Concn. of periodic acid (M)	Acetaldehyde found (μg.)	Equivalent amount of triol (μg.)	Recovery (%)
0·24	7·80	59·5	103
	7·80	59·5	103
0·12	7·61	58·0	100
	7·80	59·5	103
0·04	7·87	60·0	103
	6·69	51·0	88
0·01	5·50	42·0	72
	6·69	51·0	88

Table 3. *Effect of oxidation aeration time on yield of acetaldehyde from pregnane-3α:17α:20α-triol oxidized with 0·06 M-periodic acid*

(25·1 μg. pregnanetriol samples oxidized.)

Time aerated (min.)	Acetaldehyde found (μg.)	Equivalent of triol (μg.)	Recovery of triol (%)
5	0·11	0·9	4
10	0·45	3·4	13
15	0·62	4·7	19
20	1·13	8·6	34
25	2·29	17·4	69
30	3·07	23·4	93
40	3·32	25·2	100
50	3·27	24·9	99

Table 4. *Effect of acidity of oxidation mixture on yield of acetaldehyde from pregnane-3α:17α:20α-triol in periodate oxidation*

(25·1 μg. pregnanetriol samples oxidized.)

Normality of H_2SO_4 in oxidation mixture	Acetaldehyde found (μg.)	Equivalent amount of triol (μg.)	Recovery of triol (%)
0	0·87	6·7	27
0·01	2·31	17·6	70
0·05	2·97	22·8	90
0·1	2·76	21·1	84
0·2	3·21	24·5	98
0·4	3·35	25·6	102
0·8	3·23	24·7	98

Table 5 shows the recoveries of acetaldehyde after oxidation of pregnanetriol with 0·06 M-periodic acid in 0·4 N-H_2SO_4 with aeration for 45 min. as in the method finally adopted.

Oxidation of mixtures of acetaldehydogenic and formaldehydogenic steroids

Preliminary experiments with aqueous solutions of acetaldehyde and formaldehyde showed that a satisfactory separation of the aldehydes at levels of 10 μg. or less was possible under the conditions used for the oxidation and estimation of pregnanetriol. These conditions would be expected to permit quantitative formation of formaldehyde from formaldehydogenic steroids. It was therefore probable that the acetaldehyde and formaldehyde formed on periodate oxidation of mixtures of C_{21} steroids with various types of C-17 side chains could be separated and hence 10–100 μg. amounts of steroids estimated quantitatively.

Table 5. *Recovery of acetaldehyde on oxidation of pregnane-3α:17α:20α-triol by the method finally adopted*

(Oxidation with 0·06 M-HIO_4 in 0·4 N-H_2SO_4.)

Pregnanetriol oxidized (μg.)	Acetaldehyde found (μg.)	Triol equivalent to acetaldehyde found (μg.)	Recovery (%)
58·0	7·38	56·3	97
43·5	5·58	42·6	98
29·0	3·82	29·2	101
17·4	2·16	16·5	95
14·5	1·78	13·6	94

From mixtures of pregnane-3α:17α:20α-triol and 11-deoxycorticosterone or 11-deoxy-17-hydroxycorticosterone the amounts of acetaldehyde and formaldehyde recovered were consistent with the respective amounts of steroids present (Table 6).

Urinary studies

Collection of urine. Urine specimens were collected over 24 hr. with 1–2 ml. $CHCl_3$ as preservative and were analysed as soon after collection as possible.

Preparation of urine extracts. Extracts prepared from unhydrolysed and hydrolysed urine samples have been studied. Two methods have been used for hydrolysis of urine samples. (1) Acidification of urine: a measured portion of 50–100 ml. of a 24 hr. specimen was acidified to pH 0·95–1·1 (glass electrode) with 10 N-H_2SO_4. After 24 hr. at 25° the acidified urine was extracted with $CHCl_3$. For 'hydrolysis curves' larger portions of 24 hr. specimens were acidified to allow a number of samples to be withdrawn for analysis at various time intervals. (2) Incubation of urine with β-glucuronidase preparations: the crude β-glucuronidase was prepared (cf. Kerr & Levvy, 1951) by suspending finely minced fresh ox spleen in acetate buffer, pH 5·2, and standing at 37° for 4 hr. After centrifuging off tissue debris, the supernatant was made 60 % saturated with $(NH_4)_2SO_4$. The precipitate was dialysed free of $(NH_4)_2SO_4$, and in further fractionations the material precipitated between 25 and 50 % saturated $(NH_4)_2SO_4$ was retained. After dialysis this crude enzyme preparation was assayed, using phenylglucuronide as substrate, and expressing the activity in 'glucuronidase units' (G.U.) where 1 G.U. liberates 1 μg. phenol in 1 hr. under the conditions given by Kerr, Graham & Levvy (1948).

For incubation with enzyme, 25–50 ml. of a 24 hr. urine specimen was acidified to pH 4·5 with acetic acid and 10 ml. 0·1 M-acetate buffer, pH 4·5, added. A solution containing enzyme in the proportion of 10 000 G.U./24 hr. specimen was added and the mixture incubated at 37° for 24 hr. An enzyme blank with 25–50 ml. of water in place of urine was carried out simultaneously. Blank values have been consistently less than 10 % of the corresponding estimations.

Neutralization of urine prior to extraction. For comparative purposes it is desirable to extract urine samples after

Table 6. *Recovery of acetaldehyde and formaldehyde from periodate oxidation of mixtures of pregnane-3α:17α:20α-triol (triol), 11-deoxycorticosterone (DOC) and 17-hydroxy-11-deoxycorticosterone (S)*

Steroid oxidized (μg.)			Acetaldehyde found (μg.)	Formaldehyde found (μg.)	Steroids (μg.) equivalent to CH_3CHO and H.CHO found		Steroids oxidized (%)	
Triol	DOC	*S*			Acetaldehydogenic	Formaldehydogenic	Acetaldehydogenic	Formaldehydogenic
58·0	—	36·0	7·42	2·76	56·7	31·9	98	89
34·8	—	36·0	4·50	2·76	34·4	31·9	99	89
11·6	—	36·0	1·60	3·13	12·2	36·1	105	100
52·7	—	54·0	6·05	5·04	46·2	58·1	88	108
31·6	—	54·0	3·96	4·90	30·2	57·0	96	106
21·1	—	54·0	2·88	4·30	21·8	49·6	103	92
10·5	—	54·0	1·40	4·57	10·7	52·8	102	98
52·7	—	72·0	6·66	5·84	50·8	67·4	96	94
31·6	—	72·0	3·49	5·78	26·6	66·7	84	93
21·1	—	72·0	2·97	5·73	22·8	66·1	108	92
52·7	63·2	—	6·33	5·25	48·3	57·7	92	91
31·6	63·2	—	4·05	5·46	30·9	60·0	98	95
10·5	63·2	—	1·47	5·73	11·2	63·0	107	100
52·7	63·2	—	6·75	5·67	51·5	62·4	98	99
31·6	15·8	—	4·50	1·43	34·4	15·7	104	99
21·1	15·8	—	2·70	1·48	20·6	16·3	98	103
10·5	15·8	—	1·35	1·38	10·3	15·2	98	96
52·7	31·6	—	6·75	2·70	51·5	29·7	98	94
31·6	31·6	—	3·96	2·65	30·2	29·2	96	92
10·5	31·6	—	1·37	2·81	10·5	30·9	100	98

various pretreatments under similar conditions. In most experiments reported here, urine samples, prior to extraction, were brought to pH 6·9–7·1 (glass electrode) using 2N-Na_2CO_3 and 0·5M-K_2HPO_4. The samples were stirred vigorously during the addition of alkali.

Extraction of urine. Urine samples were extracted three times with 2 vol. of $CHCl_3$. Using these proportions, emulsions were rarely obtained. The $CHCl_3$ extracts were washed once with 0·1 vol. 0·1N-NaOH and twice with 0·1 vol. water. The water washings were washed once with 2 vol. $CHCl_3$, this $CHCl_3$ being added to the main $CHCl_3$ extract. The $CHCl_3$ extracts were dried with the minimum amount (about 2 g./100 ml.) of anhydrous Na_2SO_4 (A.R.) for 20 min. After filtering off the Na_2SO_4 the $CHCl_3$ extracts were evaporated to dryness under reduced pressure in a water bath at about 60°. The residues were transferred with three 2 ml. portions of redistilled ethanol to the oxidation tubes *A* (Fig. 1). The subsequent procedure is detailed in the Method section (p. 340).

Recovery experiments on urine extracts. The recoveries of pregnane-3α:17α:20α-triol added to $CHCl_3$ extracts of unhydrolysed, acid and enzymically hydrolysed urine were studied, in addition to the recovery of pregnanetriol added to the urine itself (added in 0·5–1·0 ml. ethanol) with subsequent extraction of the urine and purification of extracts. Recoveries were calculated from the increase in acetaldehyde obtained from extracts containing added pregnanetriol over that given by extracts without added triol.

The recoveries of pregnanetriol added to $CHCl_3$ extracts of urine where no alkali washing was carried out, varied from urine to urine (Table 7). With alkali-washed extracts recoveries were consistent, and are illustrated in the results obtained for the recovery of pregnanetriol added to urine itself with subsequent $CHCl_3$ extraction (Table 8).

The urine 'blank' values, i.e. acetaldehyde obtained from urine extracts without added triol, are a measure of the acetaldehydogenic material extractable from urine and may indicate the presence of C_{21} 17:20-dihydroxy-20-methylsteroids. The blank values given in Tables 7 and 8 are expressed as mg. pregnanetriol/24 hr.

In Table 9 are compared 'blank' values obtained from untreated urine, acid hydrolysed and enzymically hydrolysed urine from normal male subjects. The values obtained after acidification or incubation with β-glucuronidase preparations are considerably higher than those from untreated urine (cf. formaldehydogenic steroids in urine (Heard, Sobel & Venning, 1946; Paterson, Cox & Marrian, 1950; Kinsella, Doisy & Glick, 1950; Cox & Marrian, 1951). The amount of acetaldehydogenic material extractable after acidification of urine from normal males increases with time. The increase is rapid for a few hours, after which very little change is observed up to 24 hr. after acidification. ($CHCl_3$

Table 7. *Irregular recovery of pregnanetriol added to unwashed chloroform extracts of normal male urine*

		Pretreatment of urine					
		(1) None		(2) Acid hydrolysis		(3) Enzymic hydrolysis	
	Triol added (μg.)	Triol recovered (%)	Urine 'blank' (mg. as triol/ 24 hr.)	Triol recovered (%)	Urine 'blank' (mg. as triol/ 24 hr.)	Triol recovered (%)	Urine 'blank' (mg. as triol/ 24 hr.)
Urine I	0	—	0·98	—	2·15	—	3·90
	0	—	0·97	—	2·02	—	4·12
	10	19	—	102	—	35	—
	25	8	—	54	—	69	—
	50	62	—	52	—	59	—
Urine II	0	—	0·86	—	2·63	—	3·64
	0	—	0·94	—	2·79	—	3·88
	10	82	—	95	—	117	—
	25	103	—	101	—	90	—
	50	100	—	93	—	77	—

Table 8. *Recovery of pregnanetriol added to urine from normal male subjects. (Chloroform extracts alkali-washed)*

(31·5 μg. triol added to each sample.)

Subject	Urine 'blank' (value as mg. triol/day)	Recovery of added triol (%)
A	0·33	96
	(0·26)*	—
B	0·48	94
	0·49	95
C	0·40	93
	0·40	93
D	0·26	90
	0·29	93

* Loss during processing.

Table 9. *Amounts of acetaldehydogenic material found in urine of normal male subjects*

(All values expressed as mg. pregnanetriol/24 hr.)

Urine sample	Treatment of urine prior to chloroform extraction: Untreated urine	Acidification to pH 1, with immediate extraction	Acidification to pH 1; extracted after 24 hr.	Incubated at 37°, pH 4·5, with β-glucuronidase
	(*A*) Chloroform extracts alkali-washed			
I	0·33	0·44	0·92	—
II	0·28	0·43	—	—
III	0·36	—	—	1·58
IV	0·47	—	—	—
V	0·48	0·93	0·80	1·94
VI	0·12	0·19	0·29	0·66
VII	0·28	0·32	0·45	—
	(*B*) Chloroform extracts not alkali-washed			
VIII	0·92	2·96	2·80	3·60
IX	0·72	3·20	3·40	4·80

extracts were alkali-washed.) As yet, insufficient study has been made of such 'hydrolysis curves' to permit of interpretation, but they indicate the presence in urine of conjugates hydrolysed by acid at 25°. With regard to the acid stability of acetaldehydogenic steroids, it has been found that pregnane-3α:17α:20α-triol is stable to pH 1 at 25° for 24 hr.

DISCUSSION

Estimation of acetaldehyde formed by periodate oxidation of urine extracts is more specific and sensitive than the estimation of 17-ketosteroids formed (Talbot & Eitingon, 1944). As C_{21} 17:20:21-trihydroxysteroids as well as C_{21} 17:20-dihydroxy-20-methylsteroids yield 17-ketosteroids on periodate oxidation, these types of steroids are estimated together in the latter method. It is desirable for metabolic studies that these two types, which include both 21-hydroxy- and 21-deoxy-steroids, should be estimated separately. In addition to the general disadvantages of the 17-ketosteroid methods the Talbot & Eitingon (1944) method estimates the relatively small increase of 17-ketosteroids after periodate oxidation over that present before oxidation.

It has been found that neutral chloroform extracts of normal male urine contain substances which liberate acetaldehyde when oxidized with periodic acid under the same conditions necessary for quantitative oxidation of 17:20-dihydroxy-20-methylsteroids.

The presence of some types of conjugates was indicated by the increased amounts of acetaldehydogenic material extractable from urine after treatment with acid or β-glucuronidase preparations. Although C_{21} 17:20-dihydroxy-20-methylsteroids have been isolated from urine of patients with hyperactive adrenals, proof of the presence of such compounds in normal urine must await isolation studies. This work is now in progress.

Some of the hydrolysis techniques used in the study of urinary formaldehydogenic steroids have been applied to the study of acetaldehydogenic substances. These relatively mild hydrolysis conditions were employed to avoid excessive destruction of the sensitive C-17 side chain (in particular the tertiary hydroxyl at C-17). The value of enzymic hydrolysis with β-glucuronidase (especially when carried out on urine directly) for quantitative assay of urinary cortical steroids or their metabolites seems doubtful at present, when there is not adequate evidence that such enzymic hydrolysis is complete.

Acetaldehyde gives a brown coloration with chromotropic acid (McFadyen, 1945) and may interfere in the estimation of formaldehyde if present in relative excess over the latter. Where estimation of the acetaldehyde is not desired, it may be removed by aeration from the formaldehyde distillates in the Daughaday (1948) method if 1% aqueous glycine is used in place of the ethanolic sulphite solution.

SUMMARY

1. A method for estimating C_{21} 17:20-dihydroxy-20-methylsteroids in urine, based on periodic acid oxidation, is described.

2. The method allows of the simultaneous estimation of acetaldehydogenic and formaldehydogenic steroids.

3. A urinary excretion of unconjugated acetaldehydogenic material equivalent to 0·1–0·5 mg./day of C_{21} 17:20-dihydroxy-20-methylsteroids was found for seven normal males.

4. The presence of conjugated acetaldehydogenic material in normal male urine is also indicated.

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The Metabolism of Subcutaneously Injected [^{15}N] Urea in the Cat

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The experiments described in this paper arose from a study of the physiological role of gastric urease. In order to throw light on this problem, an investigation was undertaken into the fate of [^{15}N]urea in the cat.

Although Luck (1924) demonstrated the presence of urease in the stomach, urea has been widely regarded solely as an end product of mammalian nitrogen metabolism. This view was supported by experiments of Schoenheimer (1942) and Bloch (1946) who fed [^{15}N]urea to rats and found no more than a slight incorporation of ^{15}N into the proteins of liver and intestine. They believed that this could be interpreted as the result of bacterial decomposition of urea in the gut. More recently, Leifer, Roth & Hempelmann (1948) injected [^{14}C]urea into mice and recovered 20·8% of the injected ^{14}C in the carbon dioxide of the expired air. The breakdown of [^{14}C]urea in mice has been confirmed by Jones (1950) and by Skipper *et al.* (1951). Kornberg, Davies & Wood (1952) made analogous observations on cats.

In the study of the metabolism of [^{15}N]urea it must be borne in mind that the ammonia produced by hydrolysis of urea is largely reconverted to urea in the liver. Hence the amounts of ^{15}N incorporated into other nitrogenous materials give only minimum values for the urea breakdown. In order to arrive at an estimate of the actual breakdown, information is required on the conversion of [^{15}N]ammonia to [^{15}N]urea under the conditions of the experiment.

Although previous workers have studied the fate in the animal body of [^{15}N]ammonia after oral administration of [^{15}N]ammonium citrate (Foster, Schoenheimer & Rittenberg, 1939; Rittenberg, Schoenheimer & Keston, 1939; Sprinson, 1948; Sprinson & Rittenberg, 1949), and after intravenous injection of a single dose of [^{15}N]ammonium glutamate (Berenbom & White, 1950), their results did

not supply the required information. The behaviour of [^{15}N]ammonia liberated slowly and continuously into the blood was therefore investigated after periodic subcutaneous injections of [^{15}N]ammonium lactate into a cat. The amounts of urea hydrolysed, as determined by this procedure, agree with those found subsequently after injection of [^{14}C]urea into cats (Kornberg *et al.* 1952) and are far less than those found in mice (Leifer *et al.* 1948; Skipper *et al.* 1951). Part of this work has been communicated to the Biochemical Society (Davies & Kornberg, 1950).

METHODS

Preparation of labelled materials. All labelled [^{15}N] compounds administered contained 32 atoms % excess ^{15}N in the positions labelled. [^{15}N]Urea was synthesized according to Cavalieri, Blair & Brown (1948) by the interaction of [^{15}N]NH_4NO_3 (the nitrate ion being unlabelled) and diphenyl carbonate, prepared according to Hentschel (1884). The 0·31 M-[^{15}N]ammonium lactate solution was obtained by distilling [^{15}N]NH_3 from [^{15}N]NH_4NO_3 into excess lactic acid (British Pharmacopœia) and neutralizing the solution with M-$NaHCO_3$ to pH 6·5.

Treatment of cats. Cats were fed on bread, milk and water for 4 days prior to, and during, the experiment. Into cat *A* (2·00 kg.) were injected subcutaneously 98·8 mg. [^{15}N]urea in 2 ml. of water the first day, and 116·4 mg. [^{15}N]urea in 2 ml. of water the second day. The total quantity of isotope injected was 32·1 mg. ^{15}N. Cat *B* (1·20 kg.) received 28 mg. ^{15}N in a total of ten subcutaneous injections, at 4-hourly intervals, each of 2 ml. of the [^{15}N]ammonium lactate solution. Urine was collected in flasks containing 10 ml. of 10 N-HCl and 2 ml. of toluene to prevent bacterial decomposition of the constituents and loss of NH_3. On the third day of the experiment, the cats were weighed, anaesthetized with ether, and bled from the carotid arteries. The blood was collected and stored at −12°. The heads were cut off and dropped into liquid N_2; they were removed after 2–3 min. and, on being tapped gently with a hammer, split neatly along the sagittal lines. The halves were stored at −12°. The liver, spleen, kidneys, diaphragm, heart, and pieces of skin and hair were removed from each cat and frozen rapidly in solid CO_2. The stomach and intestinal tract of each animal were opened and washed under the tap before being frozen. All tissues were stored at −12°.

Analysis of urine samples. Samples (3 ml.) were pipetted into all-glass aeration units (Van Slyke & Cullen, 1916), 3 ml. of sat. K_2CO_3 were added and a rapid stream of air passed for 3 hr., to transfer the NH_3 to 0·05 N-H_2SO_4 (5 ml.). The absorbed NH_3 was estimated by titration with 0·05 N-NaOH using bromocresol green as indicator. When the end points had been reached, the NH_3 solutions were immediately acidified with 3 N-HCl and evaporated on an electric hot-plate to approx. 1 ml. They were then transferred to small ampoules which were sealed and sent off for mass spectrometric analysis.

This procedure removed 96–98 % of the [^{15}N]NH_3 present. The remainder was washed out by addition of 1 ml. of 0·214 M-NH_4Cl (containing 3 mg. N) and 1 ml. of 40 % (w/v) NaOH to the solutions, and aerating the NH_3 for 2 hr. into 0·05 N-H_2SO_4 (5 ml.). This was repeated once more: the last NH_3 samples thus obtained were always free from excess ^{15}N.

The alkaline urine samples, which were now NH_3-free, were transferred to measuring cylinders. Approximately half of the solutions were pipetted into 250 ml. beakers and neutralized to thymol blue by dropwise addition of 10 N-HCl. The urea present in each solution was then precipitated as the dixanthydryl complex (Fosse, 1916; Kiech & Luck, 1928), filtered off on weighed Royal Worcester 1/3114 filter crucibles, washed and air-dried at 110° for 3 hr. Samples (20–30 mg.) were digested by the Kjeldahl procedure. The NH_3 formed was distilled into 5 ml. of 0·05 N-HCl (Rittenberg, 1946), estimated by titration against 0·05 N-NaOH and prepared for mass spectrometric analysis as described above.

This procedure removed 95–98 % of the [^{15}N]urea present. The remainder was washed out by twice adding 2 ml. of 20 % urea to the urine samples and isolating the dixanthydryl complex. Kjeldahl and mass spectrometric analysis of the last urea samples thus obtained always showed their N to be present in the normal ratio of isotope abundance. The remaining solutions, which were now free of both [^{15}N]NH_3 and [^{15}N]urea, were digested by the Kjeldahl procedure. The NH_3 formed was estimated and prepared for mass spectrometric analysis as described above. No attempts were made to isolate the various constituents of these solutions, the N of which will be referred to as the non-urea non-ammonia N.

Independent checks of the results for the various urine fractions were obtained in all cases by a second procedure. In this, duplicate samples of 0·5–1·0 ml. of urine were pipetted into aeration units, the acid neutralized by dropwise addition of M-$NaHCO_3$ and the pH adjusted to 5·0 by addition of 0·5 ml. of 3 M-acetate, pH 5·0. Water (2 ml.) and jack bean urease solution (1 ml.), prepared as described by Davies & Kornberg (1951), were added to hydrolyse the urea, and the units left overnight. Saturated K_2CO_3 (3 ml.) was added and the NH_3 aerated into 0·05 N-H_2SO_4 (15 ml.) for 3 hr. This removed 96–98 % of the [^{15}N] (NH_3 + urea) N present in the original samples. The remainder was washed out by addition of 1 ml. of 0·214 M-NH_4Cl (containing 3 mg. N) and 1 ml. of 40 % (w/v) NaOH and drawing off the NH_3 for 2 hr. into 5 ml. of 0·05 N-H_2SO_4. This was repeated once more: the last NH_3 samples thus obtained were always found to contain N with normal isotope abundance ratio. The liquids remaining in the units, which were now free of both NH_3 and urea, were washed into Kjeldahl flasks and digested by the Kjeldahl procedure. The ammonia formed was estimated and prepared for mass-spectrometric analysis as described above, this fraction being the non-urea non-ammonia N. The two methods gave results for the isotope distribution in the various fractions of urine which agreed within 5 %.

Analysis of tissue samples. Portions (50–100 mg.) of the tissues were digested by the Kjeldahl procedure. The NH_3 formed (total N) was estimated and prepared for mass spectrometric analysis as described previously.

The liver of each cat was homogenized with 5 % trichloroacetic acid and centrifuged at 3000 rev./min. for 30 min. Portions of the supernatant liquid containing the N.P.N. were digested by the Kjeldahl procedure. The precipitate was twice washed with equal volumes of 5 % trichloroacetic acid, spun down and oven dried. The protein precipitate was boiled under reflux for 20 hr. with 10 vol. of 5 N-HCl and filtered. Excess MgO was added to portions of the filtrate, containing the amide N, and the NH_3 was drawn into 0·05 N-H_2SO_4. The solutions from which the amide N had been removed were washed twice by adding 1 ml. of 0·214 M-NH_4Cl (containing 3 mg. N) and 1 ml. 40 % (w/v)